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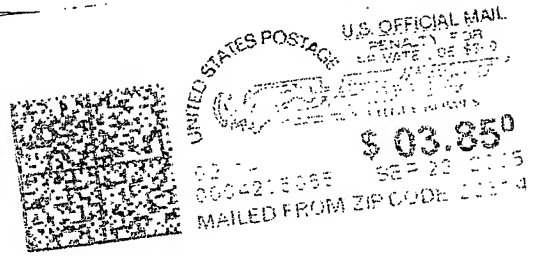

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
10/606,796	06/27/2003	Charles J. Doillon	14363	5886

7590 09/22/2005

DOWELL & DOWELL, P.C.
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EXAMINER	
BLANCO, JAVIER G	
ART UNIT	**PAPER NUMBER**
3738	

DATE MAILED: 09/22/2005

Please find below and/or attached an Office communication concerning this application or proceeding.

Office Action Summary	Application No.	Applicant(s)	
	10/606,796	DOILLON ET AL.	
	Examiner	Art Unit	
	Javier G. Blanco	3738	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *29 June 2005*.

2a)☐ This action is **FINAL**. 2b)☒ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) *1-27* is/are pending in the application.

4a) Of the above claim(s) *6,7 and 16-24* is/are withdrawn from consideration.

5)☐ Claim(s) _____ is/are allowed.

6)☒ Claim(s) *1-5,8-15 and 25-27* is/are rejected.

7)☐ Claim(s) _____ is/are objected to.

8)☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

9)☒ The specification is objected to by the Examiner.

10)☒ The drawing(s) filed on *27 June 2003* is/are: a)☐ accepted or b)☒ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

11)☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☐ All b)☐ Some * c)☐ None of:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☒ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☒ Information Disclosure Statement(s) (PTO-1449 or PTO/SB/08) Paper No(s)/Mail Date *10/07/2003*.
4) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____.
5) ☐ Notice of Informal Patent Application (PTO-152)
6) ☐ Other: _____.

DETAILED ACTION

Election/Restrictions

1. Applicants' election without traverse of **Invention I:** corneal implant (claims 1-15 and 25-27), **Polyacrylamide:** Species A (poly (N-alkylacrylamide)), and **Collagen:** Species A (telocollagen or atelocollagen) in the reply filed on June 29, 2005 is acknowledged.

2. Claims 16-24 (non-elected Invention II) and claims 6-7 (non-elected Collagen species) are withdrawn from further consideration pursuant to 37 CFR 1.142(b) as being drawn to a nonelected invention/species, there being no allowable generic or linking claim. Election was made **without** traverse in the reply filed on June 29, 2005.

Specification

3. The abstract of the disclosure is objected to because of the following informality: please substitute "ppolymer" (see line 2) with --polymer--. Correction is required. See MPEP § 608.01(b).

Drawings

4. The drawings are objected to under 37 CFR 1.83(a). The drawings must show every feature of the invention specified in the claims.

a. Therefore, the "plurality of membranes" (see claim 15) must be shown or the feature(s) canceled from the claim(s). No new matter should be entered.

Corrected drawing sheets in compliance with 37 CFR 1.121(d) are required in reply to the Office action to avoid abandonment of the application. Any amended replacement drawing

sheet should include all of the figures appearing on the immediate prior version of the sheet, even if only one figure is being amended. The figure or figure number of an amended drawing should not be labeled as "amended." If a drawing figure is to be canceled, the appropriate figure must be removed from the replacement sheet, and where necessary, the remaining figures must be renumbered and appropriate changes made to the brief description of the several views of the drawings for consistency. Additional replacement sheets may be necessary to show the renumbering of the remaining figures. Each drawing sheet submitted after the filing date of an application must be labeled in the top margin as either "Replacement Sheet" or "New Sheet" pursuant to 37 CFR 1.121(d). If the changes are not accepted by the examiner, the applicant will be notified and informed of any required corrective action in the next Office action. The objection to the drawings will not be held in abeyance.

Claim Rejections - 35 USC § 112

5. The following is a quotation of the second paragraph of 35 U.S.C. 112:

> The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the applicant regards as his invention.

6. Claims 25 and 26 are rejected under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention. Regarding claim 25, "said subject" (see line 3) lacks antecedent basis. Claim 26 depends on claim 25.

Claim Rejections - 35 USC § 102

7. The following is a quotation of the appropriate paragraphs of 35 U.S.C. 102 that form the basis for the rejections under this section made in this Office action:

> A person shall be entitled to a patent unless –
>
> (b) the invention was patented or described in a printed publication in this or a foreign country or in public use or on sale in this country, more than one year prior to the date of application for patent in the United States.

8. Claims 1, 4, 5, 8, 10, 13, 14, 25, and 26 are rejected under 35 U.S.C. 102(b) as being clearly anticipated by Perez et al. (WO 94/17851 A1).

Perez et al. disclose a corneal implant comprising a membrane (i.e., film or layer: see Abstract; see page 8, lines 16-18; page 12, lines 34-37), said membrane comprising a biological polymer (e.g. collagen type I, modified forms of collagen, glycosaminoglycans: see Abstract; page 13, lines 18-36; claims 1-5) and a hydrogel (e.g., polyacrylamide: see Abstract; page 11, lines 8-12; claims 1-5). The biological polymer to polyacrylamide ratio is disclosed at page 11, lines 31-32. The membrane thickness is disclosed at page 11, lines 32-34, and page 12, lines 34-37. Said membrane further comprises a chemical crosslink (see Abstract; see entire document). The method as claimed is disclosed in claims 20-22. It should be noted that the intended purpose of the corneal implant of Perez et al. WO 94/17851 A1 is to provide "a suitable substrate for corneal epithelial cell growth while maintaining the desirable characteristics of hydrogels, i.e., clarity, flexibility, and ability to allow diffusive flow of materials" (see Abstract; page 7, lines 26-37; page 8, lines 20-24).

9. Claims 1, 4, 5, 10-12, 25, and 26 are rejected under 35 U.S.C. 102(b) as being clearly anticipated by Graham et al. (US 5,433,745; cited in Applicants' IDS).

Referring to Figures 1-3, Graham et al. disclose a corneal implant comprising a membrane (i.e., film, coat, or layer), said membrane comprising a biological polymer (e.g. coating of a cytophilic component such as collagen, fibronectin, etc: see column 5, lines 11-35; column 10, lines 39-43) and a hydrogel (e.g., polyacrylamide: see column 3, lines 34-59). Said membrane further comprises a chemical crosslink (e.g., 1-(3-dimethylaminopropyl)-3-ethyl carboddimide or EDC: see column 6, lines 46-67; TABLE 3). The method of applying (i.e., implanting) said implant to a patient is disclosed throughout the entire document.

Claim Rejections - 35 USC § 103

10. The following is a quotation of 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

> (a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made.

11. Claims 2 and 3 are rejected under 35 U.S.C. 103(a) as being unpatentable over Perez et al. (WO 94/17851 A1) in view of Takezawa et al. (EP 0 387 975 A1).

Perez et al. disclose the invention as claimed in claims 1, 4, 5, 8, 10, 13, 14, 25, and 26 (see 102(b) rejection above). It should be noted that the intended purpose of the corneal implant of Perez et al. WO 94/17851 A1 is to provide "a suitable substrate for corneal epithelial cell growth while maintaining the desirable characteristics of hydrogels, i.e., clarity, flexibility, and ability to allow diffusive flow of materials" (see Abstract; page 7, lines 26-37; page 8, lines 20-24).

Although Perez et al. disclose the use of several polymers (e.g., polyacrylamide) suitable for the hydrogel layer, he/she did not particularly disclose using poly(N-isopropylacrylamide) [i.e., PNIPAAM] as the polyacrylamide. However, this is already known in the art. For example, Takezawa et al. disclose a prosthesis (see page 2, lines 17-18) comprising a membrane/film (see page 6, lines 48-49; see claim 9) comprising a collagen-PNIPAAM conjugate (see page 5, page 11, and page 12) in order to provide a cell growth substrate having high cell density and cellular function, and having excellent self-supporting abilities (see Abstract; see page 2, lines 3-11; see entire document). Therefore, it would have been obvious to a person having ordinary skill in the art at the time the invention was made to have combined the teaching of a prosthesis comprising a membrane/film comprising a collagen-PNIPAAM conjugate, as taught by Takezawa et al., with the corneal implant of Perez et al., in order to provide a cell growth substrate having high cell density and cellular function, and having excellent self-supporting abilities.

12. Claims 2 and 3 are rejected under 35 U.S.C. 103(a) as being unpatentable over Graham et al. (US 5,433,745; cited in Applicants' IDS) in view of Takezawa et al. (EP 0 387 975 A1).

Graham et al. disclose the invention as claimed in claims 1, 4, 5, 10-12, 25, and 26 (see 102(b) rejection above). It should be noted that the intended purpose of the corneal implant of Graham et al. US 5,433,745 is to enhance the ability to support epithelial cell growth and/or adhesion (see Abstract; see entire document).

Although Graham et al. disclose the use of several polymers (e.g., polyacrylamide) suitable for the hydrogel layer, he/she did not particularly disclose using poly(N-isopropylacrylamide) [i.e., PNIPAAM] as the polyacrylamide. However, this is already known in the art. For example, Takezawa et al. disclose a prosthesis (see page 2, lines 17-18) comprising a

membrane/film (see page 6, lines 48-49; see claim 9) comprising a collagen-PNIPAAM conjugate (see page 5, page 11, and page 12) in order to provide a cell growth substrate having high cell density and cellular function, and having excellent self-supporting abilities (see Abstract; see page 2, lines 3-11; see entire document). Therefore, it would have been obvious to a person having ordinary skill in the art at the time the invention was made to have combined the teaching of a prosthesis comprising a membrane/film comprising a collagen-PNIPAAM conjugate, as taught by Takezawa et al., with the corneal implant of Graham et al., in order to provide a cell growth substrate having high cell density and cellular function, and having excellent self-supporting abilities.

13. Claims 9, 15, and 27 are rejected under 35 U.S.C. 103(a) as being unpatentable over Perez et al. (WO 94/17851 A1).

Perez et al. disclose the invention as claimed in claims 1, 4, 5, 8, 10, 13, 14, 25, and 26 (see 102(b) rejection above). Perez et al. did not particularly disclose the claimed ratio of 0.3:1.0 (w/w) biological polymer to polyacrylamide. However, it would have been obvious to one having ordinary skill in the art at the time the invention was made to have constructed the corneal implant of Perez et al. with a particular/specific (w/w) ratio of biological polymer to polyacrylamide since it has been held that where the general conditions of a claim are disclosed in the prior art, discovering the optimum or workable ranges involves only routine skill in the art. In re Aller, 105 USPQ 233.

Regarding claim 15, intraocular and/or corneal implants comprising a plurality of membranes (i.e., layers, films, laminates, etc.) are well known in the art and would have been obvious in view of a patient's condition/disease, with the ordinary practitioner having been left

to select a particular number of membranes based on the intended purpose (e.g., different layers may provide (i) different refractive properties; (ii) site for epithelial cell adhesion/attachment; (iii) modifying the curvature of the cornea; etc.).

Regarding claim 27, packages/kits of intraocular and/or corneal implants are inherent and well known in the art. A mere arrangement of printed matter, though seemingly a "manufacture," is rejected as not being within the statutory classes. See In re Miller, 418 F.2d 1392, 164 USPQ 46 (CCPA 1969); Ex parte Gwinn, 112 USPQ 439 (Bd. App. 1955); and In re Jones, 373 F.2d 1007, 153 USPQ 77 (CCPA 1967). In re Gulack, 703 F.2d 1381, 1385-86, 217 USPQ 401, 404 (Fed. Cir. 1983)("Where the printed matter is not functionally related to the substrate, the printed matter will not distinguish the invention from the prior art in terms of patentability .... [T]he critical question is whether there exists any new and unobvious functional relationship between the printed matter and the substrate.").

14. Claims 8, 9, 13-15, and 27 are rejected under 35 U.S.C. 103(a) as being unpatentable over Graham et al. (US 5,433,745; cited in Applicants' IDS).

Graham et al. disclose the invention as claimed in claims 1, 4, 5, 10-12, 25, and 26 (see 102(b) rejection above). Graham et al. did not particularly disclose the claimed ratio of 0.3:1.0 (w/w) biological polymer to polyacrylamide, or, the claimed membrane thickness of about 20 microns to about 400 microns. However, it would have been obvious to one having ordinary skill in the art at the time the invention was made to have constructed the corneal implant of Graham et al. with a particular/specific (w/w) ratio of biological polymer to polyacrylamide, or, a particular membrane thickness, since it has been held that where the general conditions of a

claim are disclosed in the prior art, discovering the optimum or workable ranges involves only routine skill in the art. In re Aller, 105 USPQ 233.

Regarding claim 15, intraocular and/or corneal implants comprising a plurality of membranes (i.e., layers, films, laminates, etc.) are well known in the art and would have been obvious in view of a patient's condition/disease, with the ordinary practitioner having been left to select a particular number of membranes based on the intended purpose (e.g., different layers may provide (i) different refractive properties; (ii) site for epithelial cell adhesion/attachment; (iii) modifying the curvature of the cornea; etc.).

Regarding claim 27, packages/kits of intraocular and/or corneal implants are inherent and well known in the art. A mere arrangement of printed matter, though seemingly a "manufacture," is rejected as not being within the statutory classes. See In re Miller, 418 F.2d 1392, 164 USPQ 46 (CCPA 1969); Ex parte Gwinn, 112 USPQ 439 (Bd. App. 1955); and In re Jones, 373 F.2d 1007, 153 USPQ 77 (CCPA 1967). In re Gulack, 703 F.2d 1381, 1385-86, 217 USPQ 401, 404 (Fed. Cir. 1983)("Where the printed matter is not functionally related to the substrate, the printed matter will not distinguish the invention from the prior art in terms of patentability .... [T]he critical question is whether there exists any new and unobvious functional relationship between the printed matter and the substrate.").

Conclusion

15. The prior art made of record and not relied upon is considered pertinent to applicant's disclosure: Yoshioka et al. (US 6,897,064 B2).

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Javier G. Blanco whose telephone number is 571-272-4747. The examiner can normally be reached on M-F (7:30 a.m.-4:00 p.m.), first Friday of the bi-week off.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Corrine McDermott can be reached on (571) 272-4754. The fax phone numbers for the organization where this application or proceeding is assigned is 703-872-9306 for regular communications and After Final communications. Any inquiry of a general nature or relating to the status of this application or proceeding should be directed to the receptionist whose telephone number is 703-308-0858.

JGB

September 17, 2005

David H. Willse
Primary Examiner

OIPE
OCT 0 7 2003
PATENT & TRADEMARK OFFICE

INFORMATION DISCLOSURE CITATION

(Use several sheets if necessary)

Docket Number (Optional)	Application Number
14363	10/606,796
Applicant(s)	
Charles J. DOILLON et al.	
Filing Date	**Group Art Unit**
June 27, 2003	

U.S. PATENT DOCUMENTS

*EXAMINER INITIAL	REF	DOCUMENT NUMBER	DATE	NAME	CLASS	SUBCLASS	FILING DATE IF APPROPRIATE
	1.	U.S. Appl. 20010018612		Daniel R. Carson et al.			08/30/2001
	2.	U.S. 6,238,688	05/29/2001	Wu et al.			
	3.	U.S. 6,102,946	08/15/2000	Nigam			
	4.	U.S. 6,030,634	02/29/2000	Wu et al.			
	5.	U.S. 6,005,160	12/21/1999	Hsiue et al.			
	6.	U.S. 5,994,133	11/30/1999	Meijs et al.			
	7.	U.S. 5,843,185	12/01/1998	Leon Rolden et al.			
	8.	U.S. 5,661,194	08/26/1997	Ando et al.			
	9.	U.S. 5,458,819	10/17/1995	Chirila et al.			
	10.	U.S. 5,436,135	07/25/1995	Tayot et al.			
	11.	U.S. 5,433,745	07/18/1995	Graham et al.			

FOREIGN PATENT DOCUMENTS

	REF	DOCUMENT NUMBER	DATE	COUNTRY	CLASS	SUBCLASS	Translation YES	Translation NO
	12.	WO 99/37752	07/29/1999	PCT International				

OTHER DOCUMENTS *(Including Author, Title, Date, Pertinent Pages, Etc.)*

	13.	S. Shimmura et al. Biocompatibility of Collagen-Based Blended Biomaterials, Invest Ophthalmol Vis Sci 2002;43: E-Abstract 2997, pp 1-2.
	14.	May Griffith et al., Functional Human Corneal Equivalents Constructed from Cell Lines, December 10, 1999, Vol. 286: pp 2169-2172.

EXAMINER	DATE CONSIDERED
Daniel G. Pauls	9/15/2005

EXAMINER: Initial if citation considered, whether or not citation is in conformance with MPEP Section 609; Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

INFORMATION DISCLOSURE CITATION *(Use several sheets if necessary)*	Docket Number (Optional) 14363	Application Number 10/606,796
	Applicant(s) Charles J. DOILLON et al.	
	Filing Date June 27, 2003	Group Art Unit

OIPE OCT 0 7 2003 PATENT & TRADEMARK OFFICE

U.S. PATENT DOCUMENTS

*EXAMINER INITIAL	REF	DOCUMENT NUMBER	DATE	NAME	CLASS	SUBCLASS	FILING DATE IF APPROPRIATE
	15.	U.S. 5,300,116	04/05/1994	Chirila et al.			
	16.	U.S. 5,201,764	04/13/1993	Kelman et al.			
	17.	U.S. 5,114,627	05/19/1992	Civerchia			
	18.	U.S. 5,112,350	05/12/1992	Civerchia et al.			
	19.	U.S. 4,780,409	10/25/1998	Monji et al.			
	20.	U.S. 4,702,244	10/27/1987	Mazzocco			
	21.	U.S. 4,581,030	04/08/1986	Bruns et al.			

FOREIGN PATENT DOCUMENTS

	REF	DOCUMENT NUMBER	DATE	COUNTRY	CLASS	SUBCLASS	Translation YES	Translation NO

OTHER DOCUMENTS *(Including Author, Title, Date, Pertinent Pages, Etc.)*

	22.	Jean-Marc Legeais et al., Nineteen Years of Penetrating Keratoplasty in the Hotel-Dieu Hospital in Paris, 2001 Cornea 20: pp 603-606.
	23.	Jean-Marc Legeais et al., A second generation of artificial cornea (Biokpro II), Biomaterials 19 (1998) pp 1517-1522.

EXAMINER Jaime G. Pamo	DATE CONSIDERED 9/15/2005

EXAMINER: Initial if citation considered, whether or not citation is in conformance with MPEP Section 609; Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

INFORMATION DISCLOSURE CITATION *(Use several sheets if necessary)*	Docket Number (Optional) 14363	Application Number 10/606,796
	Applicant(s) Charles J. DOILLON et al.	
	Filing Date June 27, 2003	Group Art Unit

*EXAMINER INITIAL		OTHER DOCUMENTS *(Including Author, Title, Date, Pertinent Pages, Etc.)*
		Yoichi Minami et al., Reconstruction of Cornea in Three-Dimensional Collagen Gel Matrix Culture, Invest. Ophthal. & Visual Science, June 1993, Vol. 34 No. 7; pp 2316-2324.
		Teruo Miyata et al., Collagen Engineering for Biomaterial Use; Clin. Mat. 9 (1992): pp 139-148.
	26.	Toshiaki Takezawa et al., Cell Culture on a Thermo-Responsive Polymer Surface, Bio/Tech. Vol 8, September 1990, pp 854-856.
	27.	Toshiaki Takezawa et al., Morphological and immuno-cytochemical characterization of a hetero-spheroid composed of fibroblasts and hepatocytes, Journ. of Cell Science 101, 1992, pp 495-501.
	28.	Vickery Trinkaus-Randal et al., Implantation of a Synthetic Cornea, Artificial Organs 21(11): 1185-1191.
	29.	V. Trinkaus-Randal et al., Biological response to a synthetic cornea, Journ. of Controlled Release 53 (1998), pp 205-214.
	30.	S. Vijayasekaran et al., Cell viability and inflammatory response in hydrogel sponges implanted in the rabbit cornea, Biomaterials 19 (1998): pp 2255-2267.
	31.	Xin Yi Wu et al., In vivo comparison of three different porous materials intended for use in keratoprosthesis; Br. J. Ophthalmol 1998; 82: 569-576.
	32.	Traian V. Chirila, An overview of the development of artificial corneas with porous skirts and the use of PHEMA for such an application, Biomaterials 22 (2001) pp 3311-3317.
	33.	P. Giusti et al., Collagen-based new bioartificial polymeric materials; Biomaterials 1994, Vol. 15 No. 15: pp 1229-1233.
	34.	Kaarina Tervo et al., Recovery of Corneal Innervation Following Photoreafractive Keratoablation, Arch Ophthalmol/Vol 112, 1994: pp 1466-1469.

EXAMINER	DATE CONSIDERED
Daniel G. Blanco	9/15/2005

*EXAMINER: Initial if citation considered, whether or not citation is in conformance with MPEP Section 609; Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

Notice of References Cited	Application/Control No. 10/606,796	Applicant(s)/Patent Under Reexamination DOILLON ET AL.	
	Examiner Javier G. Blanco	Art Unit 3738	Page 1 of 1

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	Classification
	A	US-6,897,064 B2	05-2005	Yoshioka et al.	435/397
	B	US-			
	C	US-			
	D	US-			
	E	US-			
	F	US-			
	G	US-			
	H	US-			
	I	US-			
	J	US-			
	K	US-			
	L	US-			
	M	US-			

FOREIGN PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Country	Name	Classification
	N	WO 9417851 A1	08-1994	World Intellect	PEREZ et al.	A61M 37/00
	O	EP 0387975 A1	03-1990	European Patent	Takezawa et al.	C12N 5/00
	P					
	Q					
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NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
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*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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(21) International Application Number: PCT/US94/01419

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(30) Priority Data:
014,812 8 February 1993 (08.02.93) US

(60) Parent Application or Grant
(63) Related by Continuation
US 08/014,812 (CIP)
Filed on 8 February 1993 (08.02.93)

(71) Applicant *(for all designated States except US)*: MASSACHUSETTS INSTITUTE OF TECHNOLOGY [US/US]; 77 Massachusetts Avenue, Cambridge, MA 02139 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: PEREZ, Edward [US/US]; 52 Trowbridge Street, No. 11, Cambridge, MA 02138 (US). MILLER, David [US/US]; Francis Street, Brookline, MA 02146 (US). MERRILL, Edward, W. [US/US]; 90 Somerset Street, Belmont, MA 02178 (US).

(74) Agents: PABST, Patrea, L. et al.; Kilpatrick & Cody, 1100 Peachtree Street, Suite 2800, Atlanta, GE 30309-4530 (US).

(81) Designated States: CA, JP, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: BILAYER COMPOSITE HYDROGELS FOR CORNEAL PROSTHESES

(57) Abstract

A two-layer composite material composed of a thin layer of corneal tissue or collagen and a hydrogel, preferably formed of an electron-beam crosslinkable polymer such as a synthetic polyethylene oxide (PEO) hydrogel, is described. The material is designed to provide a suitable substrate for corneal epithelial cell growth while maintaining the desirable characteristics of hydrogels, i.e., clarity, flexibility and ability to allow diffusive flow of nutrients. In a preferred embodiment shown in the examples, the gels are synthesized via electron irradiation induced crosslinking of an aqueous solution of PEO onto a thin layer of collagenous tissue substrate. In another preferred embodiment, the gels are chemically coupled to the collagenous tissue substrate via a second polymer.

advantages

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BILAYER COMPOSITE HYDROGELS FOR CORNEAL PROSTHESES

Background of the Invention

The present invention is generally in the field of ocular materials and in particular is an improved hydrogel material for engineering of corneal surfaces.

The major anatomic divisions of the eye are the sclera, uveal tract, retina, vitreous, lens, anterior chamber, and the cornea. The clear cornea and gray-white opaque sclera are fused together and completely enclose the other portions of the eye. Under the influence of intraocular pressure, the cornea and sclera form a semi-rigid spherical organ.

The cornea occupies the anterior pole of what is referred to as the globe. In adults, the cornea measures 12 mm in the horizontal meridian and 11 mm in the vertical meridian. The central one-third of the cornea is nearly spherical and measures 4 mm in diameter. The cornea is thinner (0.5 mm) in the center than in the periphery (1.0 mm). Histologically, the cornea is composed of five layers: epithelium, Bowman's membrane, stroma, Descemet's membrane, and endothelium. The most metabolically active layers are the limiting layers, as described by Zurawski CA, McCarey BE, and Schmidt: Glucose Consumption in Cultured Corneal Cells. (1989) Current Eye Research 9(4), 349-355: endothelium and epithelium, which are primarily cellular. The epithelium serves to maintain the tear film and provide an anatomic barrier to infectious agents. In contrast to the limiting layers, the stroma is only 3-5% cellular, consisting mainly of collagen type I. As a result, 70% of dry weight of the cornea is collagen type I, with the balance being glycosaminoglycans and cellular elements. The cornea is 77% water under normal physiologic conditions. The stroma serves to mechanically maintain the geometry of the cornea, as described by Maurice D: The Cornea and Sclera. in The

Eye, Vol 1B, Davson H, editor. Florida, 1984, Academic Press, pp 1158.

Irreversible loss of corneal tissue clarity is treated by replacement of the patient's cornea, usually with a donor cornea, as reviewed by Brightbill FS, Corneal Surgery: Theory, Tissue, and Technique. The C.V. Mosby Company St Louis, Missouri (1986). In spite of the success of corneal transplants, there is a still a need for a viable prosthetic alternative. Keratoprostheses are generally indicated in cases of chemical burns, ocular pemphigoid (severe dry eyes), heavily vascularized corneas, and in cases of multiple graft failures. A viable prosthetic alternative to corneal transplants is also needed for treatment of severely intractable cornea cases as well as for cases in underdeveloped countries where human corneal tissue is unavailable due to poor donor supply and storage conditions. In addition, the appearance of AIDS and other tissue-borne diseases has caused increased scrutiny and mistrust of donor tissue even in developed countries.

When considering the synthetic replacement of the cornea, two major functional issues must be addressed. The first issue is the optical function and the second is the anatomic barrier function of the cornea. Use of synthetic materials in corneal surgery has existed, at least theoretically, since 1771 when Pellier de Quengsy conceived of implanting transparent material in the scarred cornea. In the late 1800's, glass buttons were implanted into the cornea to increase the clarity of scarred corneas. Modern devices have been designed as optically clear poly (methyl methacrylate) cylinders that penetrate the cornea and are anchored by a collar, as reported by Cardona H: Keratoprosthesis. Ophthalmology (1976) PP 284-299. These prostheses are short lived due to the progressive necrosis and stromal "melting" of the cornea close to the cylinder. The

impermeability and rigidity of these devices are believed to eventually cause the failure of the device. Since there is no complete barrier (epithelium) covering the implant, the edges of the implant also serve as a site for infection.

The complete replacement of the cornea including one or both of the limiting layers (epithelium or endothelium) is still addressed with materials such as poly (methyl methacrylate) or silicone membranes. These currently used keratoprostheses have relatively short lifetimes of between a few weeks and a few months as a result of no continuous epithelial boundary, tissue death around the implant (sterile necrosis), secondary infection (endophthalmitis), and peri-implant membrane formation.

Upon the re-evaluation of the concept of a prosthetic cornea, the issue of biological activity, i.e., cell and tissue response, of synthetic surfaces became a consideration. In either therapeutic or refractive corneal surgery, the growth of epithelium over the implant is important. Normally, the epithelium is a labile cellular population which will grow back over a denuded area of the cornea. Thus, a synthetic corneal surface must provide an environment that is conducive to epithelial cell growth.

Analysis of the surface chemistry of synthetic material surfaces conducive to epithelialization yields several basic conclusions, reported by Baier RE et al: Surface Properties Determine Bioadhesive Outcomes: Methods and Results. 1984 J. Biomed. Mater. Res. 18:337; Sugimoto Y: Effect on the Adhesion and Locomotion of Mouse Fibroblasts by their Interacting with Differently Charged Substrates. 1981 Exp. Cell Res., 135:39; Maroudas NG: Chemical and Mechanical Requirements for Fibroblast Adhesion. 1973 Nature 244:253; Salthouse TN and Matlaga: Some Cellular Effects Related to Implant Shape and Surface: in Biomaterials in

Reconstructive Surgery. 1983 Rubin, ed. (St Louis, Mosby); pp 40-45; Curtis ASG et al: Substrate Hydroxylation and Cell Adhesion. 1986 J. Cell Sci. 86:9; Petit DK et al: Quantitation of Rabbit Corneal Epithelial Cell Outgrowth on Polymeric Substrates *In Vitro*. 1990 Invest. Ophthal. Vis. Sci. 31(11) 2269-2277. Factors such as negative charge, intermediate wetabilities, moderate hydroxylation, and low polymer chain rigidity are believed to be conducive to epithelialization. These surface factors include wetability: 45° - 75°; composition: high [OH], low [COOH]; chain rigidity: low Mobility; Charge Density: Negative (2000 /10(-11) cm)^2.

In spite of identification of these factors, these investigators could not rule out the contribution of surface protein adsorption which aided in the adherence of cells. Protein or peptide attachment to surfaces is an attractive method of surface modification. Proteins which are involved in cell-substratum interaction have been shown to enhance the adherence of cells to synthetic surfaces, as reviewed by Rioslahti E, Pierschbacher MD; Arg-Gly-Asp: A Versatile Cell Recognition Signal. 1986 Cell 44, 517-518. Efforts to specifically utilize the these proteins to enhance the adherence of corneal epithelial cells has been performed with PVA copolymer hydrogels, Trinkhaus-Randall V et al: The Development of a Biopolymeric Keratoprosthetic Material. 1988 Invest. Ophthal. Vis. Sci. 29(3), 393-400. Although the initial cell-hydrogel adherence is enhanced, prolonged attachment is not maintained. This finding is probably because the methodology of coating surfaces with proteins is based fundamentally on adsorption of these proteins.

It was not until recently, as reviewed by Trinkhaus-Randall V et al: The Development of a Biopolymeric Keratoprosthetic Material. 1988 Invest. Ophthal. Vis. Sci. 29(3), 393-400, that hydrogels had

been evaluated as useful materials for a prosthetic device that would penetrate either the epithelial or endothelial side of the cornea. Hydrogels were originally conceived only as intrastromal implants. The clinical application of these intra-corneal hydrogels was to affect the shape of the cornea, as reported by McCarey BE, Andrew DM: Refractive Keratoplasty with Hydrogel Lenticular Implants. 1981. Invest. Ophthal. Vis. Sci. 21, 107-115. Initially three different hydrogel materials were used in corneal surgery: poly(2-hydroxyethyl methacrylate) PHEMA, poly(glycerol methacrylate) PGMA, and polyelectrolyte materials, described by Refojo MF: Artificial Membranes for Corneal Surgery. 1969 J. Biomed. Mater. Res. 3, 333--337. The desired characteristics of these materials were essentially their bulk characteristics such as: solute permeabilities, chemical and mechanical stability, and optical clarity. The only biological characteristics these materials had to possess is that they be non-inflammatory.

Another approach to this problem attempts to synthesize a material whose intrinsic surface is one conducive to epithelial cell growth. The synthesis of these materials utilizes crosslinking methods to form hydrogels from the collagen types (I, described by Geggel HS, Friend J, and Throft RA: Collagen Gels for Ocular Surface. 1985 Reports Invest. Ophthal. Vis. Sci. 26: 901-905, and IV, described by Thompson et al. Synthetic Collagen IV lenticules as a biomaterial for epikeratoplasty. 1990 ARVO Absts. Invest. Ophthal. Vis. Sci. 31(suppl), 301. Unfortunately, materials like these have been found to be susceptible to proteolysis *in vivo*. As reported by other investigators, Rao KP and Joseph KT: Collagen Graft Copolymers and Their Biomedical Applications in Collagen, Vol 3, Nimni, M.E., ed. (CRC press, Boca Raton Florida 1988) pp 63-85, and Franzblau C et al: Cell Growth on Collagen-HEMA

Hydrogels. id. pp 191-208, have synthesized collagen-HEMA copolymers to grow epithelial cells. These materials were also found to be susceptible to proteolysis and were brittle.

To summarize, the current modalities using proteins or peptides as materials for hydrogel-fabrication or hydrogel-surface-modification are confounded by factors such as proteolytic digestion, poor protein adherence to the synthetic surface, and brittleness or fragility. However, even considering these factors, utilizing cell-adhesion proteins immobilized on a synthetic surface is attractive since these proteins are ultimately involved in *in vivo* epithelialization.

Refractive errors are presently corrected with the use of eyeglasses or contact lenses. Another major application of synthetics in corneal surgery is the rising interest in refractive surgery, as reviewed by Thompson KP: Current Status of Synthetic Epikeratoplasty. Refractive and Corneal Surgery 7:240-248. In theory, a refractive prosthesis could affect the curvature of the corneal surface and influence the cornea's ability to focus an image on the retina thereby circumventing the need for glasses.

Implanting material onto the cornea is receiving much attention as a means of correcting refractive errors. These superficial corneal augmentations have been termed epikeratophakic grafts. Originally, epikeratophakic grafts were made from donor corneal tissue. These were not optimal, however, because of difficulty in manufacture, requiring two to three weeks post-operatively to regain clarity, and susceptibility to normal tissue remodeling processes. Synthetic epikeratophakic grafts offer ease in manufacturing and stability in clarity and geometric form. However, in the effort to develop usable synthetic epikeratophakic grafts, investigators have identified difficulties with

the synthetic materials as a result of the lack of normal regeneration of the epithelial cell layer on these materials. As a result, the development of a synthetic graft with a suitable surface for regeneration of an epithelial cell layer, which maintains the desired optical and structural properties, is required to make these devices clinically useful.

Collagen coating of conventional hard poly(methylmethacrylate) (PMMA) corneal prostheses, as described by Kirkham SM, Dangel MF: The Keratoprosthesis: Improved Biocompatibility Through Design and Surface Modification. Abstracts - 17th Cornea Research Conference, Boston (1991), increases the attachment of these devices to tissue and decreases the inflammatory responses. Still, the lifetime of these conventional, impermeable PMMA devices is short (15 months) and they are never fully epithelialized.

Hydrogel materials have received much attention in relation to their applicability as prosthetic corneal devices, as reported by Trinkhaus-Randall V et al: The Development of a Biopolymeric Keratoprosthetic Material. Invest. Ophthal. Vis. Sci. Vol. 29, No. 3 (1988) pp. 393-400; Sipehia R, et al. Towards an Artificial Cornea. Biomat., Art Cell, Art Organs; 18(5) pp. 643-655 (1990); and Thompson KP: Current Status of Synthetic Epikeratoplasty. Refractive Corneal Surg. Vol. 7 (1991) pp. 240-248, because of the properties of hydrogels, including clarity, flexibility, and ability to allow diffusive flow of nutrients. Anatomically, a penetrating corneal prosthesis must be fully enclosed in the cornea with a contiguous epithelium; i.e., contiguous epithelium must develop between the implant and host via colonization of the implant surface by epithelial cells which migrate from the host cornea. Ideally, a hydrogel material must be constructed possessing a surface environment conducive to corneal epithelial cell growth in addition to maintaining other

desirable characteristics of hydrogels. This has not yet been achieved.

It is therefore an object of the present invention to provide keratoprostheses that have longer lifetimes, and result in less tissue death, secondary infection and peri-implant membrane formation following implantation, than currently available keratoprostheses.

It is a further object of the present invention to provide a method for making and using a material with desirable optical properties, including clarity, providing an optimal epithelial cell substrate, tissue-like flexibility, and permeability to solutes, which is compatible in living systems and enzymatically stable, for use as keratoprostheses.

Summary of the Invention

A two-layer composite material composed of a thin-layer of corneal tissue or collagen and a hydrogel, preferably formed of an electron-beam crosslinkable polymer such as a synthetic polyethylene oxide (PEO) hydrogel, is described. The material is designed to provide a suitable substrate for corneal epithelial cell growth while mainlining the desirable characteristics of hydrogels, i.e., clarity, flexibility and ability to allow diffusive flow of nutrients.

In the preferred embodiment shown in the examples, the gels are synthesized via electron irradiation induced crosslinking of an aqueous solution of PEO onto a thin layer of collagenous tissue substrate. In a second embodiment, the collagenous tissue substrate, or stromal layer, is glued to a preformed hydrogel by polymerization of a liquid polymer solution, for example a polyethylene oxide solution that is polymerized by electron beam irradiation. Light microscopic studies indicate that the interface between the corneal tissue and PEO gel appears well adherent with no gaps in the interface. SEM studies of the material surface show an

architecture similar to that of normal corneal tissue. Surface analytical techniques were used to identify amino-acids covalently bound to the gel at the gel/collagen interface after the proteinaceous material was removed. ESCA survey scans identified the presence of nitrogen on exposed gel/collagen interfaces and amino acid labelling confirmed the presence of amino acids. ATR-IR studies identified increased absorption for the gel collagen interfaces at 1640 cm^{-1} and 1540 cm^{-1} indicative of bound amino acids. *In vivo* studies are being used to confirm biocompatibility, optical properties, and mechanical stability over extended periods.

Brief Description of the Drawings

Figure 1 is a micrograph of a 15% PEO gel adhered onto corneal tissue using 10 Mrad of electron beam crosslinking.

Figure 2 is an SEM micrograph of a 10 μm layer of cornea adhered onto 15% PEO hydrogel (top view).

Figure 3 is a micrograph of a cross-section of a composite lenticule grown in organ culture prepared by implantation of a composite hydrogel/stroma lenticule into a freshly enucleated rabbit eye which was grown in organ culture. The top layer of cells are confluent basal epithelial cells, overlying stromal tissue, with the hydrogel appearing as a clear portion at the bottom of the micrograph. On the surface of the basal epithelial cells are migrating epithelial cells.

Detailed Description of the Invention

A hydrogel composite whose ultimate use will be for the construction of an artificial cornea or epikeratoprostheses has been synthesized. The design rationale is to construct a material possessing a surface environment conducive to epithelial cell growth in addition to possessing the proper optical, diffusive,

and mechanical characteristics of the cornea. In the preferred embodiment, electron-irradiation-induced (EII) crosslinking is used to synthesize a hydrogel network and simultaneously attach the polymeric network to a collagenous substrate.

Described herein is a material, and methods of use thereof as a synthetic lenticule or epikeratophakic graft, synthesized of a composite material formed of a synthetic hydrogel covalently attached to a thin collagen matrix. There are essentially two embodiments, the first where the hydrogel is covalently attached directly to the collagen (or stromal) matrix and the second where there is an intermediate material which is used to adhere the collagen matrix to the hydrogel. In the presently most preferred embodiments, polyethylene oxide (PEO) or hydroxyethylmethacrylate (HEMA) and excised corneal stromal tissue are used to synthesize the hydrogel and matrix surface.

Selection and Preparation of the Hydrogel

As used herein, a hydrogel is a water swollen polymer, between two weight percent and 60 weight percent per volume of gel. Hydrogels are typically 80 to 90% water, having indices of refraction close 1.3, which is similar to that of water and, therefore the cornea. Mechanically, the hydrogels should be able to support a breaking tensile stress of between 40,000 and 60,000 dynes/cm^2. This is a relatively weak force. The polymer should be completely transparent to visible light and not absorb in the range of 300 nm to 850 nm. Chemically, the hydrogels should remain stable and not degrade *in vivo*. Small amounts of degradation product can cause an inflammatory response.

All hydrogel materials implanted into the center of the cornea have proven to be perfectly compatible and yield no deleterious effects, presumably in part because the cornea is not a vascularized organ and is therefore privileged, or isolated, from the immune system. In

other parts of the body that are vascularized, reactive functional groups on the surface of hydrogels, including amines, carboxyls, and hydroxyls, have been associated with inflammation. As described below, combining these materials with corneal stroma or collagen appears to make all of the hydrogels biocompatible within the corneal environment.

A number of polymers can be used to form the hydrogel, including hydroxyethylmethacrylate, polyethylene oxide, polyvinyl alcohol, polydioxolane, poly(acrylic acid), poly(acrylamide), and poly(N-vinyl pyrilidone). These polymers can be crosslinked by electron beams. These materials are commercially available from Polysciences, Sigma Chemical Co., and Aldrich Chemical.

In the preferred embodiment, the hydrogel is formed of crosslinked polyethylene oxide or hydroxyethylmethacrylate. Crosslinked PEO has previously been used in the synthesis of hydrogel materials for corneal implantation, as reported by Peiffer RL, Werblin TP, Fryczkowski AW: Pathology of Corneal Hydrogel Alloplastic Implants. Ophthalmology Aug 1985,92(9) p. 1294-1304. These materials have shown negligible tissue reaction and do not adsorb proteins, as reported by Merrill EW, Salzman EW: Polyethylene Oxide as a Biomaterial. Amer. Soc. for Art Int. Organs, Vol. 6, No. 2 April/June (1983) p. 60-64; Merrill EW, et al.: Nonadsorptive Hydrogels for Blood Contact. Progress in Artificial Organs, ISAO Press, Cleveland (1986) pp. 909-912.

The polymer solutions to be crosslinked range from 2 to 15 weight per volume. The preferred thickness in combination with corneal tissue or collagen is in the range of between 50 and 100 microns.

In a preferred embodiment, electron-irradiation-induced (EII) crosslinking is used to crosslink a hydrogel network onto a collagenous matrix substrate,

which serves as a substrate for cell growth. EII crosslinking is described by Dole M: The Radiation Chemistry of Macromolecules. Academic Press, new York (1972) and Shultz, AR: Radiation and Crosslinking by Radiation, in Fettes EM (ed), Chemical Reactions of Polymers. Interscience, New York 1964), the teachings of which are incorporated herein, and described in more detail in the examples below. Water is the only solvent used for electron beam crosslinking. An advantage of this method is that it can be used to polymerize and sterilize at the same time.

Other methods can be used to synthesize hydrogels. Either covalent chemical or physical bonds can be used to adhere the hydrogel to the corneal tissue or collagen. For example, endlinking of polymers with functionalized ends can be used. A hydrogel can also be formed by polymerizing monomer in the presence of crosslinking agents, such as by polymerizing acrylamide and bis acrylamide in the presence of a free radical initiator like ammonium persulfate. Endlinking and polymerizing a network is performed in a non-polar solvent such as hexane.

Preparation of the Collagen Matrix

The hydrogel is prepared in a mold shaped to provide an implant with the appropriate surface for placement in the eye and replacement of optical and physiological function, i.e., which maintains the desired geometry of the lens. The mold will typically contain a layer of corneal stroma or collagen which the hydrogel is bonded to. The hydrogel is ultimately placed on the inside of the eye, so that the epithelial cells adjacent to the implant abut the stroma or collagen and can grow over the surface of the implant. As noted above, the hydrogel will preferably be in a layer of between 50 and 100 microns. The corneal tissue or collagen layer will preferably be in a layer of between 10 and 50 microns.

A thin layer of between approximately 10 μm and 50 microns of corneal tissue is grafted to the surface of a hydrogel to form a suitable surface for cell growth and to impart added mechanical stability to the device. The tissue is preferably obtained fresh, frozen to -20°C cut on a microtome. After the tissue is placed in the mold, the tissue is lyophilized.

Corneal tissue can be obtained from the same or different species since xenogeneic tissue exchange has been shown to form viable corneal transplants. Human corneal tissue is obtained from commercial eye banks. Rabbit eyes have also been used as a source of tissue, with up to thirty films being obtained from one rabbit cornea. Bovine eyes can also be used as donor material. As noted above, the material is preferably lyophilized, which further decreases the likelihood of a reaction against the material.

The bulk of the cornea is collagen I (70% of dry weight) with the balance being glycosaminoglycans and cellular elements, as reviewed by Maurice D: The Cornea and Sclera. in the Eye, Vol. 1B, Davson H, editor. Florida, 1984, Academic Press, pp. 1-158. Collagen type I has been proven to be a suitable substrate for epithelial growth in a variety of circumstances. Collagen type I also contains peptide regions known to be involved in cell-substrate adhesion. Collagen in modified forms is a good substrate for growth of many cell types, as reported by Geggel HS, Friend J, and Throft RA: Collagen Gels for Ocular Surface. Rep. Invest. Ophthal. Vis. Sci.; 26: 901-905 (1985); Thompson KP, Hanna KD, and Gravagna P. et al. Synthetic Collagen IV lenticules as a biomaterial for epikeratoplasty. ARVO Abstracts. Invest. Ophthalmol. Vis. Sci. 1990; 31 (suppl):301. Modified forms of collagen type I are commercially available as a substrate for cellular growth. In addition to its ability to sustain cellular growth, fibrillar collagen I is not susceptible to non-

collagen type I

specific proteolytic enzymes, Oliver et al. 1872 The Fate of Cutaneously or Subcutaneously Implanted Trypsin Purified Dermal Collagen in the Pig. Brit. J. Exp. Path. 53(5), 540-549. Lastly, fibrillar corneal collagen oriented in its normal tissue architecture can provide increased tensile strength to the surface of the implant.

In an effort to utilize corneal collagen to modify the surface of a hydrogel, the first concern is the conservation of tissue architecture of collagen. In some cases the bonding is only by passive adsorption. This is a simpler approach than by chemical bonding and does not attempt to preserve the higher organizational structure of collagen *in vivo*. Collagen in corneal tissue is organized into a complex structure of fibrils oriented in parallel planes. The collagen in this tissue architecture is not easily manipulated in processing techniques unless it is solubilized, yet its soluble form no longer has the architecture it has in connective tissue.

Using the techniques described herein, the mechanical stability and substrate properties of the native structure, Azar DT, et al. "Reassembly of the Corneal Epithelial Adhesion Structures Following Human Epikeratoplasty", Archives of Ophthalmology Vol. 109, Sept. 1991 pp. 1279-1284, is exploited for better maintenance of a contiguous epithelial cell layer. This is in contrast to the prior art methods of attaching proteins onto hydrogels which bind single protein molecules or small molecular aggregates onto surface sites, reported by Weetal HH (editor), Immobilizing Enzymes, Antigen, Antibodies and Peptides: Preparation and Characterization. Marcel Dekker Inc. New York (1975), which disrupts the higher organizational structure of collagen exhibited *in vivo*. Corneal collagen is not easily manipulated in processing techniques unless it is solubilized, yet its soluble

form no longer has the architecture it has in corneal tissue, King JK: Deciphering the Rules of Protein Folding. Chem. Engin. News; 67(10) pp. 32-54 (1989). An advantage of the EII crosslinking procedure is that it does not require the solubilization of the tissue, and thus the native architecture is preserved in the final device.

In addition to corneal collagen films of less than 20 microns thick cross-linked collagen type I have been used to develop cross-linking strategies and analytical techniques.

Formation of Lenticules and Epikeratophakic Grafts

Attaching fibrillar collagen onto a hydrogel to modify the surface is dependent upon achieving a satisfactory adherence between collagen and the hydrogel. The hydrogel can be bound to the collagen matrix by mechanical or chemical coupling. Covalently bonding collagen directly or indirectly onto a synthetic hydrogel network by crosslinking provides optimal adhesion. To address both the conservation of fibrillar corneal collagen form and the interface bonding between materials, electron-irradiation-induced (EII) crosslinking is used to adhere a layer of corneal collagen to polymer molecules of a hydrogel network. In one embodiment, the electron beam simultaneously synthesizes and crosslinks the hydrogel onto the insoluble collagenous substrate. In a second embodiment, the electron beam is used to polymerize a polymer layer between the collagen matrix and the hydrogel which thereby covalently crosslinks the hydrogel to the collagen matrix. A number of polymeric materials can be used to attach the hydrogel to the collagen matrix, including any of the materials described above with reference to formation of the hydrogel itself. These materials can be the same as or different from the material forming the hydrogel.

Electron-beam irradiation has been used extensively to crosslink polymers onto hydrogels and also to attach polymers onto surfaces, as reported by, for example, Wilson JE: Radiation Chemistry of Monomers, Polymers, and Plastic. Marcel Dekker, Inc., New York, NY (1974). This method is confined to a surface attachment of polymer molecules since there is no diffusion into the collagen fibers. EII crosslinking has previously been used to form strong attachment of collagen onto a surface, as disclosed by U.S. Patent No. 3,955,012 to Okamura and Hino. Microfibrillar collagen aggregates were adhered with EII crosslinking to silicone membranes and found to be adherent in peel tests even after 10 days of washing. In contrast, the use of monomer molecules allow monomer diffusion into the collagen and affects the physical and biological properties of the material. Using a high dose rate, between 0.5 and 100 Mrads/sec, for example, for a few seconds to generate a total dose of 2 Mrads, hydrogels can be synthesized which are covalently bound to fibrillar collagen. This type of crosslinking is achieved by use of a Vandergraf generator, which generates up to a dose of 0.5 Mrads/second. The higher the dosage, the greater the amount of crosslinking. However, the dosage and extent of crosslinking must be limited to avoid damage to the underlying corneal tissue or collagen substrate, which causes, for example, the tissue to become opaque.

This crosslinking mechanism is based upon chemical activation of polymer molecules that result in the formation of polymer-polymer crosslinks to form a hydrogel and polymer-collagen, as reported by Pietrucha K, Kroh J: Radiation Crosslinking of Poly(butyl acrylate) During Polymerization and Grafted Copolymerization with Cr(III) Crosslinked Collagen. 1986 Radiation Physics Chem. 28(9),373-376; and Rao et al. Grafting of Vinyl Monomers onto Modified Collagen

by Ceric Ion -Studies on the Grafting Sites. 1969 Leather Sci. 16, crosslinks to unite the collagen and hydrogel.

The crosslinking process initiated by electron irradiation creates covalent attachment between the substrate collagen and the forming synthetic polymer network. However, less than 1% of electrons directly affect the polymer chains themselves, as reported by Stafford JW: The Irradiation Induced Reactions of Aqueous Polyethylene Oxide Solution, I. Theory of Gelation. 1970 Die Makromolekulare Chemie 134, 57-69. Hydroxyl radicals formed from the electron induced lysis of water attack hydrogens on polymers and collagen. These attacks leave radicals on the macromolecules which can recombine to form covalent crosslinks. The adherence or bonding between the insoluble collagen substrate and the hydrogel network arise from collagen amino acids crosslinked to synthetic polymer, as expected based on Pietrucha K, Lobis M: Some Reactions of OH Radicals with Collagen and Tyrosine in Aqueous Solutions. 1990 Rad. Physics Chem. 36(2), 155-160.

Solution characteristics and irradiation conditions affect the degree of polymer-collagen crosslinking. These factors can be theoretically considered by examining the major reactions, the radical generation reactions and the crosslinking reactions. The radiolysis of water is a fast reaction which creates a concentration of hydroxyl radicals, [$OH^{\cdot}$], that are consumed as fast as they are created. These hydroxyl radicals, $OH^{\cdot}$, participate in the creation of macroradicals on polymers in solution. Scission that takes place is not considered since it is first order whereas recombination of macroradicals is second order (bimolecular). Factors which increase the concentration of macroradical should also increase the amount of crosslinking. Dose rate (Mrad/sec) directly affects the concentration of hydroxyl radicals. Polymer

concentration affects the concentration of hydroxyl radicals. Polymer concentration affects the amount of collagen-polymer crosslinking by the increased availability of polymer radicals. Since the amount of collagen is fixed in a given area, only an increase in dose rate affects the concentration of collagen radicals. Since the amount of collagen is fixed in a given area, only an increase in dose rate affects the concentration of collagen radicals. The relative proportion of collagen-polymer to polymer-polymer crosslinks is governed by the ratio of rate constants $k_{p,c}$ and $k_{p,p}$ and the polymer concentration. Collagen concentration is essentially fixed per a certain area.

Formation of and Implantation of Composites

These considerations aside, the collagen or corneal tissue substrate is placed in a mold of the appropriate desired shape. For example, corneal tissue layer substrates are prepared by slicing tissue using a cryostat or microtome to a thickness between 10 and 80 microns, at a plane tangential to the surface of the cornea. The layers are placed in a pre-cooled (-20°C) glass mold in the shape of the final product. An aliquot of the polymer solution is placed onto the substrate, then exposed to an electron bean in a dose sufficient to crosslink the solution into a gel so that its final dimensions will be stable. Although the mold determines the basic shape and dimensions of the final product, this is then shaped in the same manner as other types of keratoprostheses or contact lens, trimming off material as necessary to achieve the desired curve in the cornea.

The implant is then placed in a patient's eye using standard techniques for implantation of keratoprosthesis. For example, the eye is topically anesthetized with 4% proparacaine HCl. After a few minutes, the cornea is locally anesthetized with 4% proparacaine by subconjunctival injections, and the eye

is immobilized with a lid speculum, which simultaneously keeps the eyelids open and the eye fixed, as necessary. A sterile drape is placed around the eye. Absolute ethanol is applied to the central five mm of the cornea to remove the central epithelial layer. The epithelium is further debrided from the central cornea using the blunt side of a scalpel blade. The debrided surface is then irrigated using sterile balanced salt solution (BSS) to remove any other epithelial debris. Using a five mm trephine, a superficial circular incision is made in the central cornea to a depth of 0.1 mm. A lamellar keratotectomy is performed on the central core of the cornea. A circumferential incision is then made using a rounded scalpel blade. The implant is tucked into the annular incision that has been created. The wound edges are sealed either with sutures, or, more preferably, by air-drying for three to five minutes. A pressure patch is then applied to keep the eyelid from moving over the eye surface for at least two days, to allow healing and epithelialization of the implant surface.

The present invention will be further understood by reference to the following non-limiting examples.

Example 1: Synthesis and characterization of PEO hydrogels prepared by EII crosslinking of polyethylene oxide (PEO) to a substrate of fibrillar collagen.

Light microscopy and scanning electron microscopy were used to visualize the gel/collagen interface. The materials were characterized by several chemical and spectroscopic techniques to elucidate the nature of the bonding of PEO to a substrate of collagen. Bulk proteinaceous material was removed with oxalic acid hydrolysis so the gel/collagen interface could be exposed. Electron spectroscopy for chemical analysis (ESCA) was used to determine the presence of nitrogen from amino acids on the surface. Fluorescamine amino-

acid labelling was used to confirm that the nitrogen present was in amino-acid form. Attenuated Total Reflection Infrared (ATR-IR) spectroscopy was used to determine IR spectral changes indicative of bound amino acids.

MATERIALS AND METHODS

Synthesis of Composite Gels

Collagenous substrates were either dried films of bovine collagen I (100 μm) or bovine corneal tissue (10 μm). Collagen films were made by air-drying a 10 mg/ml solution of microfibrillar collagen (Avitene, MedChem Products, Woburn, MA) placed in a 25 mm polypropylene dish. Later the films were vacuum dried for 24 hours. Sheets of corneal tissue were cut from the anterior half of the adult bovine cornea on a cryostat (Tissue Tek II) set at -22°C. The collagen substrates, from either method, were supported on glass coverslips during electron beam irradiation. An aqueous solution of 5% PEO (MW = 100K) is introduced into a 550 μl disc shaped vessel where the samples and coverslip supports are placed. Samples are irradiated from 7.5 to 10 Mrads at dose rates of 0.5 Mrad/sec. Samples of corneal tissue grafted onto PEO gels were examined under light and scanning electron microscopy. Collagen films grafted onto PEO gels were examined with analytical techniques such as ESCA, amino-acid labelling, and surface infrared spectroscopy. For characterization of the PEO gel/collagen interface, the collagen grafted onto PEO was removed through hydrolysis, using the method of Pietrucha K, Kroh J: Radiation Crosslinking of Poly(butyl acrylate) During Polymerization and Grafted Copolymerization with Cr(III) Crosslinked Collagen. Radiation Physics Chem. 28(9) pp. 373-376 (1986), with 10% oxalic acid for 150 hours to leave only amino acids covalently bound to the gel surface.

Light and Scanning Electron Microscopy

PEO hydrogels grafted onto corneal tissue were prepared into 6 μm sections and stained with hematoxylin and eosin for light microscopy. The samples prepared for SEM were fixed in glutaraldehyde and stained with OsO_4. The samples were dried in a critical point dryer (AUTOSAMDRI model 814) and visualized in a dual stage scanning electron microscope (model ISI-DS130).

Fluorescamine Labeling

The gel networks with collagen hydrolytically removed were buffered with 2 volumes of 0.2 M sodium borate buffer. One volume of fluorescamine (15 mg/100 ml acetone) was added to the mixture, giving an approximately 20-fold excess versus protein, if 0.6 $\mu g/cm^2$ of protein binds to the hydrogel surface, as reported by Udenfriend S, et al.: Fluorescamine: A Reagent for Assay of Amino Acids, Peptides, Proteins, and Primary Amines. Science Vol. 178, Nov. 24, 1972. Fluorescence of the mixture was measured in a fluorescence spectrophotometer (Perkins Elmer Model 650-10M). The instrumentation is designed quantify fluorescence for solutions. The fluorescence of gels suspended in a solution can be tested but the determination of the gel surface amine concentration is not readily ascertained.

ESCA

This analysis was performed on gels with collagen hydrolyzed from the surface. The samples were also air dried overnight and vacuum dried for six hours. The samples were analyzed in an Surface Science spectrophotometer (Mod 101). Survey scans focused on spot sizes of 1000 μm.

ATR-IR

The samples were prepared by air drying the materials overnight and then vacuum drying for six hours. The material were studied in a Perkins Elmer IR spectrophotometer (Model 1430) utilizing a ZnSe crystal

for multiple internal reflectance. The range of study was from 4000 cm^{-1} to 700 cm^{-1}.

Results

Microscopy

Initial visualization of the PEO gel/tissue interface was performed through light microscopy and SEM. Light micrographs of corneal tissue grafted onto PEO gels with a 10 Mrad dose of irradiation are shown in Figure 1. These samples were embedded in paraffin, sectioned at 6 μm and stained with hematoxylin and eosin. The gels appear well adherent with no gaps in the interface between gel and tissue at the light microscopic level.

Scanning electron microscopy of gels grafted onto 10 μm sections of corneal tissue provided a more detailed visualization. The top view of the cut edge of the material is shown in Figure 2. Lamellae characteristic of cornea are seen overlying the hydrogel which is beneath the tissue. One to two corneal lamellae are seen in the micrograph. Comparison with a micrograph of the typical appearance of corneal tissue, from Komai Y, Ushiki T: The Three-Dimensional Organization of the Collagen Fibrils in the Human Cornea and Sclera. Invest. Ophthal. Vis. Sci. 32:2244-2258, 1991, shows that the crosslinked corneal tissues looks the same as normal corneal tissue.

ESCA and Fluorescamine Labelling

The objective of the ESCA and fluorescamine labelling was to verify that stable covalent bonding between the gel and collagen had occurred during EII crosslinking and to characterize the nature of the bonds formed. Aqueous irradiation forms carbon radicals on the amino acid which recombine with other carbon radicals produced on the polymeric network to form PEO collagen crosslinks. The carbon-carbon bonds are very stable but peptide hydrolyzable with acidic media. Only the carbon-carbon bonds remain intact following

hydrolysis of these peptide bonds, leaving single amino acids bound to the PEO network via carbon-carbon bonds. Control surfaces were prepared by carrying out the same hydrolysis reaction in the presence of corneal collagen which was simply placed in contact with the gel, but which had not been crosslinked.

ESCA was used to investigate the presence of residual amino acids on the surface. Survey scans of materials included those for PEO gels alone and with grafted collagen removed. The survey scan showed the presence of carbon, oxygen, sodium, chloride and nitrogen. The ratio of carbon to oxygen is roughly 2:1, indicative of the high concentration of PEO on the surface. Nitrogen is assumed to arise from the presence of amino acids bound to the surface of the hydrogels. PEO crosslinked without collagen was also exposed to the hydrolysis. After washing, these gels do not exhibit the presence of nitrogen on the surface.

Fluorescamine labelling was used to assay for nitrogen present in amino acid form. Primary amine-fluorescamine conjugates fluoresce at 475 nm with excitation at 390 nm. Fluorescence was noted for those collagen grafted gels which were treated hydrolytically. PEO gels not crosslinked to collagen did not fluoresce.

Surface Infrared Spectroscopy

Surface analysis for functional groups was performed with ATR-IR on PEO gels with collagen removed by acid hydrolysis. Three control spectra were obtained for comparison: (1) Linear PEO (100 K), (2) Collagen Type I, and (3) Pure PEO gel treated with acid hydrolysis. Characteristic absorption frequencies are shown in Table 1, as shown in Yannas IV: Collagen and Gelatin in the Solid State. J. Macromolecular Sci.-Revs. Macromol. Chem., C7(1) (1972) p.49-104

The collagen material gave an IR spectrum with characteristic peaks at 1640 cm^{-1} and 1540 cm-1 due to Amide I and Amide II bands. This compares identically

with published data for collagen, Weetal (1975). The non-crosslinked linear PEO shows characteristic absorption peaks of C-H vibrations and the distinctive peak at 1080 cm^{-1} due to the C-O ether linkages, Vandeberg JT (editor): An Infrared Spectroscopy Atlas for the Coating Industry. Federation of the Society for Coating Technologies, Philadelphia, PA (1980). Electron beam crosslinked PEO shows the appearance of carbonyl and carboxyl functionalities at 3080 cm^{-1} and 1535 cm^{-1}, Dennison KA: Radiation Crosslinked Poly(ethylene oxide) Hydrogel Membranes. PhD Thesis, MIT (1986).

Table 1: Characteristic Infrared Absorption Frequencies

Bond	Wavenumber (cm^{-1})
O-H	3450-3400
N-H (stretch) Overtone of Amide II	3330-3290
C-O (Amide I)	3060-3100
N-H (deformation) C-N (Amide II)	1550-1535
CH_2 Scissor	1445-1455
CH_2 Wag	1340-1310
CH_2 Stretch N-H Deformation (Amide III)	1270-1230
C-O Ether linkages and Hydroxyl	1085-1075

IR spectra elicited for collagen on PEO gel confirms the presence of collagen on the surface of the gel. After the hydrolytic removal of collagen from the surface of the gels, the IR spectra regressed to a form similar to that of non-grafted PEO gels also treated hydrolytically. The peak at 1085-1075 cm^{-1} identifies the major presence of PEO on the surface. The

significant differences in the infra-red spectrum of pure crosslinked PEO with gels which had collagen hydrolytically removed are the increased absorption at the Amide I, Amide II regions, and below 1000 cm^{-1}, Silverstein, et al., "Spectrometric Identification of Organic Compound", 4th Ed. John Wiley and Sons, Inc. New York (1981). There is a higher absorption for gels grafted onto collagen with 10 Mrad doses than those for 7.5 Mrad doses indicating a dose response behavior.

Light micrographs showed the material to be unified bilayer materials with no discernable gaps at the interface. SEM shows that corneal tissue adhered onto the gels appears similar in architecture to normal tissue although further studies of tissue architecture are needed. By hydrolyzing the collagen off of the grafted PEO gels, only amino acids covalently bound to the gels were left on the surface. ESCA survey scans identified the presence of nitrogen on the surface of the gels. Furthermore, fluorescamine labelling of these gels identified the nitrogen as present in amine form. Although the presence of amino acids was verified with labelling, the number and type of amino-acids present was not readily ascertained. To complement these surface studies of the gel/collagen interface ATR-IR was used. The IR spectra also identified the presence of amino-acids with accentuation of bands at 1640 cm^{-1} and 1540 cm^{-1}. In addition, the IR spectra identified a higher number of amino-acids covalently bound as a function of crosslinking dose.

Because complete epithelialization of the surface of the device is needed for long-term clinical function, a material which provides both a suitable surface for cell attachment and which allows for diffusion of nutrients from the anterior chamber of the eye to the surface of the prosthetic cornea was developed. Previously, electron induced covalent bonding of collagen to non-ophthalmic medical materials has been

performed in an effort to increase the adherence of collagen, as reported by US Patent No. 3,955,012 (May 4, 1976) by Okamura S and Hino T: Silicone Rubber Coated with Collagen. Strong, stable adhesion of the corneal tissue layer to the gel is necessary so that the synthetic surface modification for biocompatibility is permanent. The stability of covalent bonding further ensures that the thin-layer tissue will remain on the synthetic material truly imparting the corneal tissue biological activity, as reviewed by Brightbill FS, Corneal Surgery: Theory, Tissue and Technique. The C.V. Mosby Company St. Louis, Missouri (1986), and strength to the implant.

These results demonstrate that collagen can be covalently linked to PEO hydrogels by EII-crosslinking, and that the bonds are stable toward acid hydrolysis. Furthermore, the collagen retains features of its native architecture and may be a more suitable substrate for corneal epithelial cell growth than other formulations of collagen.

Example 2: Synthesis and Physical characterization of hydrogel bonded to collagen by EII.

The simultaneous synthesis and chemical attachment of hydrogels onto a collagenous matrix is performed using an electron beam source, a collagen substrate layered onto the bottom of a container (Fluoroware, H22 Series), and an aqueous solution of 5.25% polymer covering the collagen substrate. The assembly (container, collagen, and solution) are irradiated with doses of 10 Mrads at a rate of up to 100 Mrad/sec. The resulting hydrogels grossly appear to be optically clear, firmly gelled, and adherent to the collagen.

Substrates of corneal tissue, 70% collagen (pre-extracted) and 100% collagen (extracted), are made into sheets from freshly enucleated bovine (calf) corneas. The collagenous substrate is placed in a freezing microtome, and sheets of tissue, 10 mm in diameter, can

be cut into thicknesses varying from 20 microns or larger. The semi-rigid collagen sheets are placed into 25.4 mm diameter air-tight polypropylene dishes. After lyophilization, substrates are kept in a vacuum at 0°C until use.

Aqueous solutions of polyethylene oxide (PEO) or polyvinyl alcohol (PVA) are used in the synthesis since hydrogels made of these polymers are well-characterized (see Table 2). The polymer solutions are placed over the collagen sheets in polypropylene dishes which are now ready for electron beam irradiation. The high voltage source of electron irradiation is supplied by a Van de Graft generator. The irradiation area is 1 cm^2. This machine is capable of delivering dose rates of up to 10 Mrad/sec to samples conveyed along a belt at 1 cm/sec. Gelling and crosslinking is virtually instantaneous in these systems. After irradiation, the samples are ready for further analysis.

Table 2: PEO/PVA Hydrogel Characteristics

Characteristics	Poly(ethylene oxide)	Poly(vinyl alcohol)
Ultimate strength	app. 25 psi	180-400 psi
Polymer concentration	5-25%	10-25%
Permeability*		
albumin	negligible	7.6-3.18
inulin	4.1-2.8	not available
Clarity	+	+
E-beam crosslinked	+	+

* cm sec x 10^{-6}

Important factors in polymer-collagen crosslinking are polymer concentration, polymer morphology, irradiation dose, dose rate, and depth of penetration. By varying the parameters stated above, polymer-collagen interfacial crosslinks can be varied.

Interfacial Analysis

In order to observe the interface with surface analysis techniques, the collagen is removed by enzymatic and oxalic acid degradation, using the method of Pietrucha (1986). This preserves the gel structure while still hydrolyzing the protein into amino acids. Collagen is first cleaved by collagenase, then lysed into peptides by non-specific protease and finally hydrolyzed into amino acids (AA) by oxalic acid. Proteolytic degradation removes all AAs except those which are chemically bonded onto polymer chains of the hydrogel. Non-extracted amino-acids (from collagen) are considered covalently linked onto the hydrogel surface and are detectable by surface analytical techniques and amino acid labeling techniques. The amino acids which are most highly reactive to participation in crosslinking are shown in Table 3.

Table 3: Reactive Amino Acids in Collagen

Amino Acid	1000 No. of AA residues	$k_2 \times 10^8$ ($mol^{-1}sec^{-1}$)
Proline	126	2.0
HydroxyProline	92.1	3.0
Arginine	49	10.0
Phenylalanine	14.2	7.0
Histidine	5.4	70.0
Tyrosine	3.6	10.0

Interface topology is studied with screening methods such as scanning electron microscopy (SEM). The architecture of hydrogels in the hydrated state is preserved by certain processing methodologies or use of environmental scanning electron microscopy. SEM-identified interface topology will verify any auxiliary mode of attachment such as mechanical intercalation of

hydrogel into the collagen matrix. This may show up as roughness in the hydrogel interface.

A quantitative surface analysis technique is electron spectroscopy for chemical analysis (ESCA). Immobilized AAs analyzed in ESCA provide binding energy signals of nitrogen in addition to polymeric carbon and oxygen from PEO. The nitrogen (N1S) peaks from ESCA can allow quantification of the amount of elemental nitrogen on the top 50 angstroms of the surface. This is an indirect measure of the quantity of surface bound AAs. ESCA can analyze surface areas from 5 mm to 150 micrometers to a depth of 1-20 atomic layers. The precision of elemental analysis varies from 10-20%. Elemental detection limits are from 0.1-1.0%. The quantity of bound AAs and its relationship to polymer concentration, irradiation dose, dose rate, and irradiation depth of penetration is to be ascertained.

An additional method of studying interface amino acids bound to synthetic polymer is to label these amino acids with fluorescent or colored labelling molecules. Amino acid labelling reagents have been used classically to label the amino termini of peptides being sequenced. Some of these labelling reagents are: ninhydrin, fluorescamine, dansyl chloride, and orthophthaldiol aldehyde (OPT), which can be obtained from Sigma Chemical Co., St. Louis, MO. The labelling protocols which use these reagents can detect nanogram to picogram quantities of the amino acids. Also, the particular absorbance characteristic of labelled amino acids allow the identification and quantification of particular amino acids. Polymer/amino acid conjugates can be acid hydrolyzed from the hydrogel network and spectrophotometrically quantified.

In order to determine sites of activation, separation of the AAs can be performed with high pressure liquid chromatography. This technique can be used to find attachment sites of polymer to AAs.

Standards for such analyses can be made by electron beam irradiation of AA homopolymers within polymeric solutions. Standard amino acid homopolymers linked to polymer chains are hydrolyzed into single AAs. The networks and bound amino acids are washed to remove any residual amino acids not bound. The polymeric network is then hydrolyzed to free amino acid/monomer dimers. These dimers can be labelled with colored or fluorescent derivatives and separated through HPLC. Comparison of labelled elution fractions of samples against standard elution profiles derived from homopolymeric amino acids allows identification and quantification of modified AAs. Some irradiation modification of amino acids is expected but the majority of radical site generation will be on the alpha carbon of the peptide chain.

Light microscopy is used to analyze the histologic appearance of the collagen in the composite material. Immunofluorescence techniques are highly sensitive in antigenically determining biological changes. Antibodies to specific portions of the collagen type I molecule are commercially available. To perform immunofluorescent analyses, composites are sectioned and stained with the appropriate antibodies. Changes in the staining properties are used as a indirect measure of changes in the biological character of fibrillar collagen.

An *in vitro* enzymatic degradation assay can allow determination of the process induced changes which alter the proteolytic susceptibility of the collagen in the composite material. Unmodified collagen is not susceptible to non-specific proteases and mildly sensitive to mammalian collagenase. Test materials are incubated in a particular enzyme for 20 hours and the amount of hydrolysis is compared to that of corneal collagen. Differences in degradation rate between hydrogel/collagen composites and cornea can signal changes induced by the process of composite synthesis.

Attenuated total reflection Fourier transform infra-red spectroscopy (ATR-FTIR) utilizes infrared absorption to observe the chemical functionalities at a surface. This technique is not a true surface analytical technique although it penetrates the surface of the material to a depth of approximately one micron. It has been used to observe the surfaces of fibrillar collagen and gelatin. The ability of ATR-FTIR to determine fingerprint spectra of collagen allows one to follow the fundamental chemical changes induced in collagen due to irradiation induced crosslinking.

Example 3: Analysis of biological surface activity and growth of corneal epithelial cells onto composite.

Epithelium formation was examined following surgical implantation of the composite hydrogen through organ culture of rabbit cornea. The ability of the hydrogel to support the outgrowth of epithelial cells in a physiologic fashion, i.e., from the remaining corneoscleral rim, was demonstrated by placing the entire cornea into culture. Tissue explants were also studied to evaluate the migration and growth potential of corneal epithelial cells on the lenticule. Tissue explants provide cells which closely resemble normal cell populations in the body. In the assay, corneal tissue is placed epithelial side down on the lenticule, so that donor corneal epithelium is in direct contact with the adherent corneal stromal layer of the lenticule. Epithelial cells migrate off the tissue explant and onto the lenticule surface. Subsequently, the cells multiply and form an epithelium. Physiologically, corneal epithelial cell migration is important in normal healing. When the cornea is injured during surgical procedures, the regrowth of the epithelial layer is typically the limiting factor in healing, and the return of function is dependent upon the regeneration of a normal epithelium.

Adhesion of stratified epithelium to the underlying stroma is ultimately dependent upon the generation of numerous subcellular components in the basal layer, including hemidesmosomes, the basal lamina, and anchoring fibrils. Sipehia, et al., Biomat., Art Cells, Art Org. 18(5), 643-655 (1990). Studies have shown that compared with successful epikeratoplasty implants, implants that fail are found to have a decreased number of hemidesmosomes, a decreased area of basement membrane, and a decreased percentage of basement membrane area occupied by hemidesmosomes. In addition, irregularities and duplications of the basement membrane have been noted, as well as a diminished penetration into the corneal stroma by anchoring fibrils in those lenticules that ultimately fail, as reported by Azar, et al., Ophthal. 109, 1279-1284 (Sep. 1992). To date no one has demonstrated the presence of hemidesmosomes in *in vitro* studies of corneal epithelial migration on synthetic materials. Therefore, in this study, transmission electron microscopy was used to search for evidence of basal specialization obtained by migrating epithelial cells on our lenticule.

Materials and Methods

Characteristics of the Hydrogel: The implant under study was a composite of 2% polyethylene oxide hydrogel, 150 μm in thickness, with a 50 μm layer of native corneal tissue covalently attached. It was sterilized with 70% ethanol prior to implantation.

Organ Culture: New Zealand White rabbits were sacrificed with an overdose of sodium pentobarbital administered intravenously. The eyes were immediately enucleated and transported on ice in a moist chamber to the laboratory. Each globe was rinsed for 1 min. with normal saline containing 200 μg/ml polymyxin B sulfate and prepared and draped in a sterile fashion. An epithelial wound was created by marking an area on the

central cornea with a 5 mm trephine cut to a depth of approximately 1 mm and removing the encircled epithelium by blunt dissection with a Paton spatula. The debrided surface was irrigated with sterile phosphate buffered saline (PBS). Next a peripheral lamellar dissection was performed at the bottom of the trephine cut with an angled lamellar dissector extending to the limbus, as described by McDonald, et al., Kaufman, 823-845 (1988). Using Polack forceps and a sterile cotton swab the implant (previously cut to a diameter of approximately 10 mm) was placed on the denuded cornea and the edges were tucked below the recipient lip so that they lay in the peripheral lamellar keratotomy created by the lamellar dissector. Due to the large area of lenticule lying within the circular keratotomy, no sutures were required to mechanically secure the lenticule. During surgery, the cornea was periodically moistened with sterilized saline solution containing polymyxin B. The cornea was then carefully excised, including a scleral rim of 2 mm, and the explant was brought into culture by transfer into a 6 well plate (Costar) containing sufficient media to cover the tissue. The media was prepared from keratinocyte serum-free media (Gibco) supplemented with epidermal growth factor (2.5 μg), BPE (25 mg), L-glutamine, and gentamicin (pento 20 μg). The plates were then incubated at 37°C and 5% CO_2 for 5 to 7 days with a media change after three days.

Following the incubation period, the eyes were immersed in 10% paraformaldehyde for fixation. The corneas were imbedded in methacrylate and three μm cuts were made by microtome for histologic examination. The sections were stained with hematoxylin-eosin (H&E) and toluidine blue for light microscopy and evaluation of cell morphology and differentiation.

Tissue Explant Studies: New Zealand white rabbits were killed with an overdose of sodium pentobarbital administered intravenously. The corneas were excised

and rinsed twice in sterilized PBS. The endothelial cell layer of each cornea was removed with a sterile cotton swab and four circular disc-shaped buttons (5 mm in diameter) were obtained by sterile trephin from just within the corneoscleral junction. The buttons were placed, epithelial side down, on the synthetic lenticule in a 6 well tissue culture plate and covered with 3.0 ml of NBK-5A media. The culture dishes were allowed to incubate at 37° C and 5% CO2, with a media change after 2 days. After 7 days, the lenticules were immersed in half-strength Karnovsky's glutaraldehyde-formaldehyde fixative, post-fixed in 2% osmium tetroxide, stained en-bloc with uranyl acetate, dehydrated and embedded according to standard technique. Semi-thin sections were cut and stained with para-phenylenediamine (PPDA) for phase contrast microscopy. Ultra-thin sectioned and uranyl acetate/lead citrate stained tissues were viewed with a Philips 410 transmission electron microscope.

Results

Organ Culture: Histologic examination revealed migration of epithelial cells onto the hydrogel within a seven-day period. A confluent layer of epithelial cells was observed over the implant over time with scattered areas of two cell layered stratification.

Tissue Explant Studies: Transmission electron microscopy (TEM) revealed well-adherent, healthy appearing epithelial cells attached to the stromal layer of the lenticule. Numerous mitochondria could be seen to predominate in the basal layer.

Purpose

The goal of this project is to evaluate epithelial cell interactions with a synthetic material designed for keratoprostheses.

Methods

Composite hydrogels are hybrid materials made of corneal stromal tissue and a synthetic polyethylene oxide hydrogel base. The hydrogel is coated with a 40

μm layer of corneal stromal tissue bound to the surface of a hydrogel. Epithelial wound closure was evaluated on these materials using a two chamber cell culture system meant to mimic the clinical situation. The hydrogels are suspended between a humidified air chamber (5.5% CO_2) and a serum free media chamber. Epithelial cell migration and proliferation onto the material is observed daily via phase contrast microscopy. Samples are processed by immunohistochemistry to determine production of basement membrane proteins.

Results

Epithelial cells from corneal tissue explants readily adhere and grow to confluence over the composite hydrogel surface. Three millimeter diameter wound areas are epithelialized within four days after an initial two day lag period. Histological results show a multilayered epithelium 2 to 3 cell layers thick. By week 8, immunohistochemistry demonstrates the deposition of a continuous basal layer of laminin.

Conclusions

Composite hydrogels of stroma and synthetic hydrogel support epithelial cell attachment and growth to confluence. Laminin deposition and multilayer stratification demonstrates the ability of the materials to support regeneration of epithelial characteristics.

Modifications and variations of the present method and materials for forming and using epikeratoprostheses will be obvious to those skilled in the art from the foregoing detailed description. Such modifications and variations are intended to come within the scope of the appended claims.

We claim.

1. An implantable composite keratoprosthesis comprising

a biocompatible hydrogel bonded to a layer of a collagen material selected from the group consisting of collagen type I and corneal tissue, wherein the hydrogel-collagen material is in the shape of a corneal implant having the collagen material on the surface where it will abut the epithelial cells surrounding the implant.

2. The keratoprosthesis of claim 1 wherein the hydrogel is between two weight percent and 60 weight percent per volume of gel, has an index of refraction optically equivalent to that of water and able to support a breaking tensile stress of between 40,000 and 60,000 dynes/cm^2.

3. The keratoprosthesis of claim 1 wherein the hydrogel is between approximately 50 and 100 microns in thickness and the collagen material is between approximately 10 and 50 microns in thickness.

4. The keratoprosthesis of claim 1 wherein the hydrogel is selected from the group consisting of polyethylene oxide, polyvinyl alcohol, polydioxolane, hydroethylmethacrylate, poly(acrylic acid), poly(acrylamide), and poly(N-vinyl pyrilidone).

5. The keratoprosthesis of claim 1 wherein the hydrogel is covalently bonded to the collagen material.

6. The keratoprosthesis of claim 1 wherein the hydrogel is polymerized and bound to the collagen material by electron irradiation induced crosslinking.

7. The keratoprosthesis of claim 1 wherein the keratoprosthesis is formed by lyophilizing the collagen material in a mold and bonding the hydrogel onto the surface of the collagen material.

8. The keratoprosthesis of claim 1 wherein the hydrogel is bound to the collagen material by

polymerization of a material which couples to both the hydrogel and to the collagen material.

9. The keratoprosthesis of claim 1 wherein the hydrogel is mechanically coupled to the collagen material.

10. A method for making an implantable composite keratoprosthesis comprising

forming the composite by bonding a biocompatible hydrogel to a layer of a collagen material selected from the group consisting of collagen type I and corneal tissue, in the shape of a corneal implant having the collagen material on the surface where it will abut the epithelial cells surrounding the implant.

11. The method of claim 10 wherein the hydrogel is between approximately 50 and 100 microns in thickness and the collagen material is between approximately 10 and 50 microns in thickness.

12. The method of claim 10 wherein the hydrogel is selected from the group consisting of polyethylene oxide, polyvinyl alcohol, hydroxyethylmethacrylate, polydioxolane, poly(acrylic acid), poly(acrylamide), and poly(N-vinyl pyrilidone).

13. The method of claim 10 wherein the hydrogel is covalently bonded to the collagen material.

14. The method of claim 10 wherein the hydrogel is polymerized and bound to the collagen material by electron irradiation induced crosslinking.

15. The method of claim 10 wherein the keratoprosthesis is formed by lyophilizing the collagen material in a mold and bonding the hydrogel onto the surface of the collagen material.

16. The method of claim 10 wherein the hydrogel is adhered to the collagen material by absorption.

17. The method of claim 10 wherein the hydrogel is bound to the collagen material by polymerization of a material which couples to both the hydrogel and to the collagen material.

18. The method of claim 10 wherein the hydrogel is mechanically coupled to the collagen material.

19. The method of claim 10 further comprising trimming the collagen material surface of the implant to provide the desired optical properties.

20. The method of claim 10 further comprising performing a lamellar keratotectomy in the eye of a patient in need of treatment and implanting the prosthesis into the patient's eye.

21. The method of claim 20 wherein the prosthesis is attached by air drying.

22. The method of claim 20 wherein the prosthesis is attached by suturing.

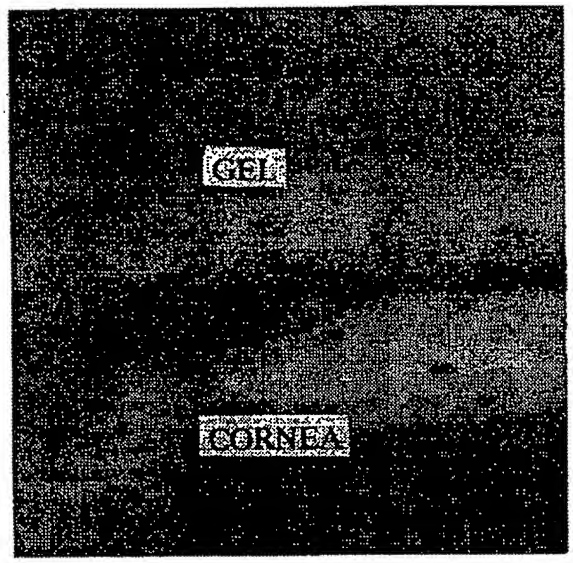

FIG.1

FIG.2

FIG. 3

INTERNATIONAL SEARCH REPORT

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A. CLASSIFICATION OF SUBJECT MATTER
IPC(5) :A61M 37/00
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According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 128/898; 156/1, 60, 272.2; 606/107; 623/4-6, 901

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

NONE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	US, A, 5,067,961, (KELMAN ET AL.), 26 November 1991. See the entire document.	1, 2, 4-10, 12, 13, 16-18 --- 1-22
Y	US, A, 5,196,027, (THOMPSON ET AL.), 23 March 1993. See entire document.	19-22

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
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(54) Cell culture substrate cell sheet, cell cluster and preparations thereof.

(57) The invention provides cell culture substrates, cell sheets, cell clusters and preparations thereof using temperature-responsive polymeric compounds.

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CELL CULTURE SUBSTRATE, CELL SHEET, CELL CLUSTER AND PREPARATIONS THEREOF

Technical Field

This invention relates to a cell culture substrate. More particularly, this invention relates to a carrier suitable for cell culture where cell passage is required.

This invention further relates to a cell sheet and/or to a cell cluster and to the fabrication method of such a cell sheet and/or cell cluster. More particularly, this invention relates to a cell sheet and/or a cell cluster which have excellent self-supporting abilities and cellular functions and the method of fabrication of such a cell sheet and/or cell cluster. The cell sheet and/or cell cluster of this invention are useful for efficient production of cell products. They are also useful as a prosthesis which is used for repairing the damaged or diseased part of living tissue or as a simulating system to evaluate the effect of drugs and so on, on a living body.

Background Art

The major applications of the current mammalian cell culture technology are 1) bioreactors for production of cell products with physiological activities, 2) prosthesis for diseased or damaged living tissues or organs, 3) simulators to evaluate toxicity and activity of drugs and so on. The outline of the prior technology concerning the mammaliam cell culture is as follows.

The mammalian cells used in cell culture technology can be divided into two types. They are anchorage independent cells and anchorage dependent cells.

The former group, the anchorage independent cells, are cells which can perform their cellular functions, such as viability, proliferation and ability to produce substances, without a substrate that serves as a foothold for the cells. Typical examples are hybridomas formed from myeloma cells and lymphoma cells.

In contrast, the latter group, the anchorage dependent cells, are cells which normally cannot perform their cellular functions such as viability, proliferation and ability to produce substances, without a substrate that serves as a foothold for the cells. The majority of normal diploid cells, including primary cells, are anchorage dependent. Even many of the established cell lines are known to show anchorage dependency. For example, the established cell lines for production of useful cell products including cytokines, such as interferon and interleukin, differentiating factors such as erythropoietin, colony-stimulating factor, thrombopoietin, and tissue plasminogen activator and vaccines, are known to be anchorage dependent. In addition, most of the cells which are applicable to prosthesis for a diseased or damaged living body and to a simulator for evaluation of toxicity and activity of drugs, are conceived to be anchorage dependent. Therefore, establishment of culturing technology for the anchorage dependent cells is extremely important for these applications.

In general, to utilize cells for these applications it is important to culture the cells in a large quantity and at high density while keeping the cells at full functional level. However, animal cells, more so than microbial cells, are highly susceptible to the effects from deficiency of supply of nutrients such as oxygen, and to the effects of accumulation of metabolic waste products.

In the case of anchorage independent cells, suspension culture techniques are considered to be the best. If cells are cultured in suspension with agitation, waste materials can be removed quickly and nutrients can be supplied efficiently, and therefore it is easier to scale up the equipment for mass and high density culture.

However, in the case of anchorage dependent cells, it is not possible to use the suspension culture technique because the cells require a substrate for attachment. Therefore, different cell culture devices with substrate for cell attachment have so far been developed. For example, in experimental scale, dish-type, flask-type and plate-type devices have been most widely used. However, the above mentioned devices are not suitable for mass cell culture. Therefore, different ideas were conceived to increase the surface area of the substrate where the cells could adhere, relative to the total volume. For example 1) roller bottle type where bottles for cell culture are rotated to grow the cells on the entire surface of the wall, 2) multiple tray type where plates for cell adhesion are arranged parallel in the culture medium and the culture medium is circulated among the plates, 3) coil type where a plastic film formed into a coil is inserted into a cylindrical tube which is rotated in a lateral manner to adhere the cells, and then a culture medium is circulated among the film, 4) hollow fiber type where hollow fiber membranes possessing semipermeability are allowed to come in contact with the cells on the external surface of the hollow fibers and the culture medium is

circulated through the interior of the hollow fiber to supply nutrients and remove waste materials through the hollow fiber membranes, 5) packed glass bead type where cells are in contact and adhered to the packed glass beads and the culture medium is circulated among them, 6) microbead type where microbeads are suspended in the culture medium to attach the cells on the surface of the microbeads which are agitated to culture the cells.

As mentioned above, prior attention has been mainly paid to morphological design of the cell culture device from the view point of effectiveness in nutrient supply and in waste removal. Recently however, it has been found that it is almost impossible to maintain cell viability and functions for a long period only by controlling the efficiency of nutrient supply and waste removal, but the cell culture substrate is a key to control the cell viability and functions for anchorage dependent cells. Therefore, research on the relationship between the property of cell culture substrate and cell functions has been actively carried out.

In the past, polystyrene is most widely used as a material of cell culture substrate because of its optical transparency, non-toxicity, excellent mechanical properties, good moldability and low price. However, the cell adhesion process which leads to the cell proliferation process is significantly inhibited on the surface of the polystyrene culture substrate because of its hydrophobicity. Therefore in order to improve the cell attachment and proliferation, the modified hydrophilic polystyrene which is endowed with negative charges by corona discharge treatment, has been developed and widely used as a cell culture substrate. However, it was found that the above-mentioned modification of polystyrene is still not enough for cells to express and maintain their specific functions.

Recently, the study to bring the cell culture substrate closer to the in vivo environment around the cell has started in order to improve cell functions such as attachment, proliferation, differentiation, and production ability of cell products. Namely, the study is to incorporate the substances which effectively control the cell functions into the cell culture substrate. The most typical substance to control the functions is extracellular matrix. Study of the function of extracellular matrix in vivo has progressed rapidly in recent years. It has become clear that it plays, not only a simple passive role such as supporting the cells and fixing the cells as known in the past, but also has a function in actively controlling or regulating cell functioning. Although a number of extracellular matrix components have been identified, the most important component is collagen. In addition it has been discovered that there are more than ten different types of collagen each of which is synthesized by a certain definite cell and is located in a certain tissue playing the role of controlling different cell functions. Even with the same type of collagen, modification by introducing a variety of functional groups or modification of higher order structure can cause different effects on the cell functions. As well as collagen, extracellular matrix components such as fibronectin, laminin, thrombospondin, vitronectin, proteoglycan and glycosaminoglycan have been identified. These have specific binding sites relative to the collagen and cell membrane and also play an important role in the cell attachment and proliferation.

Furthermore, except for the above-mentioned extracellular matrices, there are some other substances which effectively control cell functions such as attachment, proliferation and differentiation. They are gelatin which is a thermally degenerated collagen, lectins which bind specifically to sugar moiety on the cell membrane, anchorage oligopeptides which are the binding sites of anchorage proteins such as fibronectin, and adhesive protein isolated from a shellfish.

As examples of the culture substrates combined with these substances which control the cell functions, collagen-coated substrate (K. Yoshizato, et al., Annals of Plastic Surgery, 13, 9, 1984), fibronectin-coated substrate (F. Grinnell, Expl. Cell Res. 102, 51, 1976) and the substrate coated with adhesive protein of a shellfish (P. T. Picciano, et al., Developmental Biology 22, 24, 1986) have been developed, and some improvements in cell attachment and proliferation have been found.

Furthermore, recently the culture substrate coated with polystyrene containing galactose-derivative group as a side chain has been developed and some improvements in the attachment and life of hepatocytes have been recognized (T. Akaike, et al., Jpn. J. Artif. Organs, 17, 227, 1988). By using the cell culture substrates mentioned above, recently it has become possible to culture the cells which have not been able to attach and proliferate on the prior culture substrate such as glass or polystyrene.

However, despite these advancements in culture devices and substrates, the current cell culture technology has the following crucial problems still.

The distinct feature of the culture of anchorage dependent cells is that the cells stop further proliferation if the cells proliferate and completely cover the surface of the substrate. This is called contact inhibition. Therefore, the passage process, that is, the process to detach the cells from the old substrate and then to transfer the detached cells to a new substrate is necessary in order to continue the proliferation. In the past, proteolytic enzymes such as trypsin and collagenase, and EDTA as a calcium chelator were most commonly used for the cell detachment process. However, the prior cell detachment process, like

trypsinization, not only causes significant damage to the cell function, but also is a crucial obstacle to the cell culture process. The problems are as follows:

1) Prior detaching agents destroy not only the bonds between cells and the culture substrate but also bonds between neighboring cells. Three types of intercellular bonds, that is, tight junction, gap junction, and desmosome are known. The tight junction plays the role of barrier to the permeability of substances between the apical and basal sides. Through the gap junction, the exchange of substances and information is carried out between the neighboring cells, and by the desmosome, the cell assembly is mechanically supported. The cell is not able to be alive and functional alone, but the intercellular junctions enable the cell to express and maintain specific functions (B. Alberts, et al., "Molecular Biology of the Cell", 3rd edn., Garland Publishing Inc., New York & London, P. 673, 1983). Accordingly, the prior detaching agent causes crucial damage to the functions of the cultured cells by destroying completely the intercellular junctions formed in the culture process at the time of passage.

2) On the cell membrane there are many receptors for signaling molecules such as hormones, local chemical mediators, and neurotransmitters and the target cell communicates with the secreting cell through the specific reaction between the receptor and the signaling molecule. It has been found that the prior detaching agents destroy the receptors (e.g. C. Sung, et al., Biochem Pharmocol, 38, 696, 1989). Accordingly, the cells treated with the prior detaching agents cannot be controlled by the signaling molecules. This means that the cell loses its specific functions.

3) As a nutrient, the common culture medium contains serum which holds potent trypsin inhibitors. Therefore, prior to trypsinization the cells have to be washed thoroughly with a buffer solution in order to remove the trypsin inhibitors. This washing procedure not only complicates the operation, but also causes contamination which is a lethal problem in cell culture technology.

These major problems mean that even if the cells with the specific functions can be cultured by use of the sophisticate culture substrate combined with the extracellulrr matrix and also the effective design of a culture device for supply of nutrients and removal of waste, the recovery process of the cultured cell by use of the prior detaching agents markedly damages the cellular functions. Particularly, the deterioration of cell functions induced by the prior cell recovery process significantly reduces the ability of production of cell products. Also, by the cell recovery method, the self-supported cell assembly for a prosthesis cannot be acquired because the cell detaching agents such as trypsin completely break the cell assembly. Furthermore, the cells treated with the current recovery process are not applicable to simulators for evaluation of activity of the drugs, since the membrane-bound receptors responsive to the drugs are completely digested by trypsin. In addition, the prior recovery process brings a crucial shortcoming particularly to the mass cell culture technology. Excessively low initial cell concentrations of anchorage dependent cells in culture medium is said to retard the proliferation of cells and the ability to produce substances even if the cells adhere to the substrate. Particularly for the cases with primary cells or normal diploid cells which are difficult to harvest, cultivation in a large volume of culture media from the start will reduce the cell concentration excessively, and therefore the concentration of the cells has to be increased by repeating the cell culture in steps using a culturing device having a smaller capacity. This fact means that in the mass cell culture process, a lot of repeated cell recovery processes are necessary. Accordingly, the effect of the prior cell detachment procedure is such more crucial compared to a small quantity cell culture process.

The objective of this invention is to provide a cell culture substrate which can solve the problems such as deterioration of cellular functioning, complication of operation, and risk of contamination, which accompany the cultured cell recovery process by use of prior cell detaching agents such as trypsin, collagenase, EDTA and so on.

Another objective of this invention is to provide a cell sheet and/or a cell cluster which are recovered without cell function damage caused by prior detachment agents. These cell sheets and/or cell clusters are available for production of cell products, prosthesis for diseased or damaged living tissues or organs and a simulator to evaluate toxicity and activity of substances such as drugs.

Description of the Invention

The cell culture substrate of this invention comprises a temperature-responsive polymeric compound that has a lower LCST than the cell culture temperature. Here, LCST or lower critical solution temperature, is a transition temperature for hydration and dehydration of the polymeric compound. The cell culture substrate of this invention can additionally comprise substances which effectively control cell functions such as attachment, proliferation and differentiation.

The cell culture substrate of this invention can solve the problems such as deterioration of cell

functions, risk of contamination and laboriousness of operation that accompany cell recovery and passage which are the problems of the cell culture technique of the prior art.

This invention provides: 1) a substrate made from a temperature-responsive polymeric compound having lower LCST than the culture temperature; 2) a carrier formed by coating the said polymeric compound on the supporting material; 3) a carrier made by graft-polymerizing the said polymeric compound on the surface of the supporting material; 4) a carrier which consists of microbeads made from the said polymeric compound having a crosslinked structure; 5) a substrate made from a mixture of the said polymeric compound and substances which effectively control cell functions such as attachment, proliferation and differentiation; 6) a carrier formed by coating the mixture of the said polymeric compound and substances which effectively control cell functions on the supporting material; 7) a carrier formed by laminating a support with the said polymeric compound layer and a layer of substances which effectively control cell functions in sequence; 8) cell sheets and/or cell clusters formed by any of the foregoing.

The temperature-responsive polymeric compound having lower LCST than the culture temperature to be used as substrate in this invention is in a solid state which the cells can utilize as an anchor to adhere and proliferate at cell culture temperature, and will become a soluble state by reducing the temperature below the LCST to permit detachment of cells from the substrate for passage. In addition, in a carrier grafted with the temperature-responsive polymeric compound, the exchange between hydrophilic and hydrophobic states induced by temperature change will detach the cells. In a microbead carrier made from the temperature-responsive polymeric compound with crosslinked structure, the exchange between hydrophilic and hydrophobic states, and between swelling and deswelling states will detach the cells.

Examples of temperature-responsive polymeric compounds that can be used as a substrate in this invention are poly-N-substituted (meth)acrylamide derivatives and their copolymers, polymethylvinyl ether, polyethylenoxide, etherized methylcellulose, and partially acetylated polyvinyl alcohol. Particularly preferred compounds are poly-N-substituted acrylamide derivatives or poly-N-substituted methacrylamide derivatives or their copolymers.

For example, poly-N-isopropylacrylamide (PNIPAAm) is a polymeric compound which shows a negative temperature coefficient of solubility in water (Heskins, M., et al., J. Macromol. Sci.-Chem., A2(8), 1441, 1968). The hydrate (oxonium hydroxide) which depends on the hydrogen bonding formed at a lower temperature between a water molecule and the polymer molecule will decompose at a higher temperature, so that polymers aggregate by dehydration to form a precipitate. Thus, the transition temperature of this hydration and dehydration is called "lower critical solution temperature" or LCST. Thus above the LCST, the said polymer aggregates to form a solid state. But at a temperature lower than the LCST, the polymer dissolves in water.

The present invention takes advantage of such properties of the temperature-responsive polymeric compounds and completes a substrate for cell culture that can attach or detach the cultured cells by merely changing its temperature.

Appropriate temperature-responsive polymeric compounds to be used as substrates of this invention are indicated below, but this invention is not limited to these examples. The LCST of these polymers rise with the sequence of polymers listed below.

Poly-N-acryloyl piperidine, poly-N-n-propyl methacrylamide, poly-N-isopropyl acrylamide, poly-N,N-diethyl acrylamide, poly-N-isopropyl methacrylamide, poly-N-cyclopropyl acrylamide, poly N-acryloyl pyrrolidine, poly-N,N-ethylmethyl acrylamide, poly-N-cyclopropyl methacrylamide, poly-N-ethyl acrylamide.

The aforesaid polymers may be homopolymers or copolymers with other monomers. Any hydrophilic monomers or hydrophobic monomers can be used as the monomer for copolymerization. Generally speaking, copolymerization with hydrophilic monomer will raise the LCST, and copolymerization with hydrophobic monomer will depress the LCST. With a proper selection of monomers, a copolymer with a desired LCST can be achieved.

Examples of hydrophilic monomers are N-vinylpyrrolidone, vinylpyridine, acrylamide, methacrylamide, N-methyl acrylamide, hydroxyethyl methacrylate, hydroxyethyl acrylate, hydroxymethyl methacrylate, hydroxymethyl acrylate, acrylic acid and methacrylic acid having acidic groups and its salts, vinyl sulfonic acid, styrylsulfonic acid and N,N-dimethylaminoethyl methacrylate, N,N-diethylaminoethyl methacrylate, and N,N-dimethylaminopropyl acrylamide having basic groups and their salts, but it is not limited to these compounds.

Examples of hydrophobic monomers are acrylate derivatives and methacrylate derivatives such as ethyl acrylate, methyl methacrylate and glycidyl methacrylate and so on, N-substituted alkyl (meth) acrylamide derivatives such as N-n-butyl (meth) acrylamide and so on, vinyl chloride, acrylonitrile, styrene, and vinyl acetate and so on, but it is not limited to these compounds.

In general, a copolymer with a monomer having a basic group is desirable because it may enhance

adhesiveness of cells to the substrate by electrostatic interaction with the negatively charged cell membrane.

On the other hand, in this specification, the substances which effectively control cell functions such as attachment, proliferation and differentiation refer to the extracellular matrix, gelatin, lectins, anchorage oligopeptides which are the binding sites of anchorage proteins such as fibronectins, and adhesive protein isolated from a shellfish and so on. The extracellular matrix refers to the substances existing among cells within the living body. Specifically collagen, fibronectin, laminine, vitronectin, proteoglycan, glycosaminoglycan and thrombospondin and so on are included.

In addition to such properties of temperature-responsive polymeric compounds, the present invention focuses on the substances which effectively control cell functions and has perfected a cell culture substrate that can anchor and proliferate specific cells with a high degree of cell functioning by combining the said polymeric compound and the cell controlling substances.

There is no particular limitation with the shape of the substrate of this invention, and it can be of various shapes such as film, sheet, granule, fiber, flake, sponge, microbeads and so on. As a substrate for the cell culture, film, sheet, granules or fibers are particularly desirable. In the case of microbeads, the preferred particle size is 50-300 microns.

Ordinary molding methods of polymeric compound can be used to form these shapes. For example, a polymer with or without substances such as extracellular matrix is dissolved in water or organic solvent and the solution is formed into a film or sheet by a standard solvent casting technique. The liquid polymer mixture, while being cooled below the LCST, is extruded through an orifice into water or water-immiscible organic solvent at a temperature higher than the LCST, to form a film, grain, particle or fiber. The polymer may be formed into any desired shapes by suspension polymerization, precipitation polymerization, or molding directly into grains. To fabricate microbeads the polymer is dropped in water-immiscible organic solvent to form microbeads which are then insoluble.

To fabricate a carrier of this invention, a supporting material having a desired shape is prepared ahead of time, and then the polymer, with or without substances such as extracellular matrices, is coated on the surface of that supporting material by a standard method. The method of coating a thin layer of an aqueous or organic solution of the said polymeric compound, with or without substances such as extracellular matrices, on the surface of the supporting material by solvent casting methods and drying that film is particularly simple and effective. To produce a carrier that has a layer of polymeric compound and a layer of substances such as extracellular matrix on the support, the carrier can be prepared by a method by which each layer is coated in a sequential manner.

For graft-polymerizing the polymeric compound on the surface of a supporting material, one can select a polymeric compound having a desired LCST and a copolymerization grafting technique with various types of monomers can be used to regulate it to any desired LCST. When performing copolymerization graft technique, hydrophilic monomers or hydrophobic monomers can be used. In general, copolymerization with hydrophilic monomers causes the LCST of the graft copolymer to rise. Conversely, copolymerization with hydrophobic monomers will depress the LCST. Types of hydrophilic monomers and hydrophobic monomers that can be used in this invention are listed above.

Materials which have been conventionally used as substrate for cell culture are desirable as the supporting material in the case of graft-polymerizing the polymeric compound onto the surface. Examples are glass, polystyrene, polycarbonate, polymethyl methacrylate, polystyrene, polypropylene, polyethylene, polyesters, polyamides,, polyvinylidene fluorides, polyoxymethylene, polyvinyl chloride, polyacrylonitrile, polytetrafluorethylene, polydimethylsiloxane, cellulosic polymers, crosslinked dextran, crosslinked polyacrylamides, collagen and so on, but they are not limited to these compounds.

An appropriate method of grafting the temperature-responsive monomer to the supporting material can be selected based on the types of materials used and their shape.

membrane

Low temperature plasma polymerization technique can be used appropriately when the supporting material is a sheet, film or flat membrane. This method can graft-polymerize only on the surface of the supporting material without damage to the bulk property and is applied to polymers which have difficult forming radicals by usual methods such as polypropylene, polyethylene, polytetrafluoroethylene, polydimethylsiloxane, polyesters, polycarbonates, polymethyl methacrylate and so on. If the supporting material is a hollow fiber membrane or microbeads, the ozone oxidation method and cerium ion method are most appropriate. Particularly, these methods are most suitable as a graft-polymerization method for the hollow fiber membrane made of cellulosic polymer, or for microbeads made of crosslinked dextran, crosslinked polyacrylamide, or collagen.

A carrier prepared by graft-polymerizing the said polymeric compound on the surface of a support can have various shapes based on the cell culture method and property of the support and so on. Examples are

1) sheets or films, such as culture dishes, 2) hollow fiber membranes or flat membrane types, and 3) microbeads, and so on.

Besides, graft polymerization methods using ultraviolet light, x-ray, gamma-ray or electron beam can be used.

Crosslinking structures can be formed by using a method of introducing the crosslinking structure during polymerization of the monomer or by using a method of introducing the crosslinking structure after completion of polymerization. Either method can be used in this invention.

Specifically, the former method is carried out by copolymerizing bifunctional monomers. For example, N,N-methylenebisacrylamide, hydroxyethyl dimethacrylate or divinylbenzene can be used. With the latter method, it is common to crosslink the molecules by light, electron beam and gamma-irradiation.

On the other hand, the present invention also takes advantage of the properties of temperature-responsive polymeric compounds and has completed a cell sheet and/or cell cluster. After forming a cell monolayer on the substrate of the temperature-responsive polymeric compound with or without substances such as the extracellular matrix by culturing cells at a higher temperature than the LCST, the cell sheet can be prepared by detaching it from the substrate by lowering the temperature below the LCST. In order to recover a cell sheet formed on the prior substrate, cell detaching agents such as trypsin are necessary. The prior cell detaching agents destroy not only junctions between cells and the substrate but also junctions between individual cells. Accordingly, it was impossible to prepare an excellent self-supporting cell sheet by the prior art. Furthermore, the prior cell detaching agents significantly damaged the cell membrane and membrane-bound receptors.

On the other hand, in this invention it is possible to detach and recover a cell sheet from the substrate by merely changing the temperature instead of using the prior detaching agents. This invention first enables the formation of an excellent self-supported cell sheet which was almost impossible to make by the proir detaching agents. Also, this invention can keep viability and cell functions of the cell sheet due to no use of the prior detaching agents. This invention also enables the formation of an excellent self-supported cell sheet which was almost impossible to make by the prior detaching agents. Furthermore, this invention can significantly simplify the prior complex cell detaching process where the cell washing process and trypsin adding process are necessary. This means that this invention can markedly reduce the possibility of contamination which is a lethal problem in cell culture technology.

The cell detached from the substrate by the method in this invention were sheet-like immediately after the detachment, but if the cell sheet was transferred to a non-anchorage hydrophobic dish, the cell sheet gradually rolled up and finally changed to a cell cluster. It is a matter of course to be able to keep sheet-like without changing to a cluster if the circumference of the cell sheet is fixed during the detachment process.

The cell density of the thus formed cluster can be in the order of 10^9cells/ml. This cell density is about 100 times higher than the maximum cell density that can be attained by prior culture (order of 10^7cells/ml). Thus, although it is a simple calculation, it means that the scale of the equipment for production of cell products can be reduced to about a hundredth.

In the prior art, it was impossible to control the size of the cell cluster and also to produce the cluster in mass, since the prior cell cluster was formed by accidental detachment from the substrate (Koide, N. et al., Jpn. J. Artif. Organs 17 (1), 179, 1988). In this invention, however, it is easy to control the size of the cluster by changing the surface area of the culture substrate where the cell proliferated and covered before detachment and also to make the cluster in mass. In this invention, the size of the cluster ranges from several microns to several mm. Particularly, this invention is more suitable for production of the larger clusters which were not able to be prepared by the prior art. The larger clusters are effective as the prosthesis for diseased or damaged living tissue or organs.

In addition, when the cell cluster was transferred to a new hydrophilic dish, after the cell cluster was preserved on a non-anchorage hydrophobic dish in a CO_2 incubator for a long period using a common culture medium, the cell cluster started to reattach and reproliferate on the hydrophilic dish. This evidence shows that the cell cluster can keep its viability and cell function for a long period. This is because the intercellular junctions and membrane-bound receptors of the cell cluster are kept intact by the detachment method in this invention.

The cell culture substrate of this invention enables a very simple cell recovery by replacing the prior cell detaching agents with mere change in temperature and reduce the complexity and risk of contamination of the prior cell culture technology. In addition, the cell sheet and/or cell cluster of this invention showed high cell density, high cellular functioning and excellent self-supporting properties. This means that the cell sheet and/or cell cluster of this invention are strongly available for 1) bioreactors for production of cell products, 2) prosthesis for diseased or damaged living tissues or organs, and 3) simulators to evaluate toxicity and activity of drugs and so on.

The following abbreviations have been used throughout in describing the invention.

AIBN - 2',2'-azobisisobutylnitrile
cm^2 - square centimeter
CO_2 - carbon dioxide
°C - degrees centigrade
DMEM - Dulbecco's modified Eagle's medium
FCS - fetal calf serum
g - gram
LCST - lower critical solution temperature
ug - microgram
ul - microliter
ml - milliliter
mm - millimeter
mM - millimolar
n-BMA - n-butyl methacrylate
PBS - phosphate buffered solution
% - percent
PNIPAAm - poly-N-isopropyl acrylamide
THF - tetrahydrofuran

Examples and comparative examples are illustrated below to explain further and embody this invention.

Example 1

N-Isopropyl acrylamide monomer (NIPAAm, Eastoman Kodak Co.) 50g was dissolved in 500ml benzene. Using 0.2g AIBN as the polymerization initiator, polymerization was carried out at 60° C for 12 hours in a stream of nitrogen gas with agitation. The obtained polymer precipitated in benzene and after decantation the precipitation was dissolved in THF and purified using ethyl ether. LCSTs of the thus obtained PNIPAAm in different solutions (polymer concentration: 1%) which were measured by turbidimetric method are shown in Table I. As shown in Table I, it was found that PNIPAAm shows the sharp conversion between hydrophobic and hydrophilic states not only in water but also in calf serum and culture medium.

Table I

LCST of PNIPAAm in different solutions	
Solution	LCST (° C)
Distilled Water	31.8 ± 0.1
PBS	28.9 ± 0.1
Calf Serum	28.2 ± 0.1
Culture Medium	28.6 ± 0.1

Example 2

NIPAAm 50g, n-butyl methacrylate (n-BMA) 3.3g and AIBN 0.21g as the polymerization initiator, were dissolved in 500ml of THF. Polymerization was carried out at 50° C for 12 hours in a stream of nitrogen gas with agitation. After concentrating the reaction mixture twofold in an evaporator, it was precipitated and purified with ethyl ether and dried under vacuum to obtain a flaky polymer (Cop.(NIPAAm/BMA)-1). In addition, by using NIPAAm 50g, n-BMA 6.6g and AIBN 0.21g, Cop.(NIPAAm/BMA)-2 was obtained by the same method as the Cop.(NIPAAm/BMA)-1. LCSTs of the obtained polymers in PBS and calf serum were measured by turbidimetric method and are listed in Table II. As shown in Table II, with the comonomer ratio

of n-BMA, the LCST of the copolymer significantly decreased.

Table II

LCST of Cop(NIPAAm/BMA) in PBS and calf serum		
	LCST (°C)	
Polymer	PBS	Calf Serum
PNIPAAm	28.9 ± 0.1	28.2 ± 0.1
Cop.(NIPAAm/BMA)-1	19.0 ± 0.1	20.5 ± 0.4
Cop.(NIPAAm/BMA)-2	7.8 ± 0.3	13.8 ± 0.2

Example 3

By dissolving PNIPAAm and NIPAAm monomer which were synthesized and used in Example 1, in DMEM containing 10% FCS, 1.0% PNIPAAm and 1.0% NIPAAm, solutions were prepared for cytotoxicity tests. For control, DMEM containing 10% FCS was used. Then, human dermal fibroblasts were dispersed in PNIPAAm, NIPAAM and control solutions so as to form a cell density of about 1 x 10^5/ml and each solution of 2 ml was poured into the plastic 35 mm cell culture dishes (Falcon Co.). The cells were cultured at 25° C in a CO_2 incubator (air/5% CO_2). After culturing for one or three days, attachment and proliferation of the cells were examined by a phase contrast microscope. The degree of attachment and proliferation were used as a measure of cytotoxicity. The results are shown in Table III. As shown in Table III, no cytotoxicity of PNIPAAm was observed, although strong cytotoxicity was recognized in the NIPAAm monomer.

Table III

Cell attachment and proliferation		
	Cell attachment and Proliferation	
Substance	1-day culture	3-day culture
Control	○	◎
PNIPAAm	○	◎
NIPAAm	×	×
◎ : Excellent ○ : Good × : Poor		

Example 4

The cytotoxicity of Cop.(NIPAAm/BMA)-1 which was synthesized in Example 2 was evaluated by the same method as Example 3 and shown in Table IV. Here, the cell culture was carried out at 17° C so that the polymer can dissolve in the culture medium. As shown in Table IV, no cytoxicity was observed in Cop.-

(NIPAAm/BMA)-1.

Table IV

Cell attachment and proliferation		
	Cell Attachment and Proliferation	
Substance	1-day culture	3-day culture
Control	Δ	O
Cop.(NIPAAm/BMA)-1	Δ	O
Mixture of NIPAAm and n-BMA monomers	×	×
O : Good Δ : Not Very Good × : Poor		

Example 5

An aqueous solution (0.5%) of PNIPAAm which was synthesized in Example 1 was prepared and was sterilized by autoclaving (121 °C, 20 minutes) and then cooled to redissolve the polymer. To coat, after pouring the 0.5% aqueous PNIPAAm solution into the plastic 35 mm cell culture dish (Falcon Co.) and coating uniformly, excess solution was discarded and the dish was dried in a clean hood at room temperature. The above-mentioned process was performed asceptically. Human dermal fibroblasts which were used in Example 3 were dispersed in DMEM containing 10% FCS to form a cell density of about 2 x 10^5/ml. Two mililiters of the cell suspension kept at 37 °C was poured into the PNIPAAm-coated dish which was kept at 37 °C. The cells were cultured at 37 °C in a CO_2 incubator (air/5% CO_2). After culturing for 7 days, colony formation was sporadically found on the bottom of the dish and the outside of the dish was cooled to 15 °C. By a phase contrast microscope it was observable that the colony which attached on the dish spontaneously detached from the bottom.

Example 6

An aqueous solution (0.5%) of Cop.(NIPAAm/BMA)-1 which was synthesized in Example 2 was prepared and was sterilized by autoclaving (121 °C, 20 minutes) and then cooled to redissolve the polymer. Using the solution, Cop.(NIPAAm/BMA)-1-coated dish was prepared by the same method as Example 5. Then the human dermal fibroblasts were cultured on the coated dish and the colony formation similar to Example 5 was found after 7 days. When the dish was cooled to about 10 °C, the colony was found to spontaneously detach from the dish surface.

Example 7

An aqueous solution (0.5%) of PNIPAAm which was synthesized in Example 1 was prepared and sterilized by filtration through a 0.45 micron filter. This solution was mixed with an equal volume of 0.5% Type I collagen solution which was solubilized from cow skin by pepsinization (Sterilized, Koken K.K.) to prepare a solution containing 0.25% PNIPAAm and 0.25% collagen as the final concentrations. LCST of the mixture solution which was measured by turbidimetric method was about 32 °C. This solution was poured into the plastic 35mm cell culture dish (Falcon Co.) and dried in a clean hood at room temperature. The

above-mentioned process was performed asceptically. Thus, dishes which were coated with a mixture of collagen and PNIPAAm in equal volume in different thicknesses were prepared. Here, the coating thickness was controlled by the volume of the mixture solution poured into the dish. Then, the human dermal fibroblasts which were used in Example 5 were dispersed in DMEM containing 10% FCS to form a cell density of about 2 x 10^5/ml. Two milliliters of the cell suspension kept at 37° C was poured into the dishes coated with the mixture of PNIPAAm and collagen in different thicknesses which were kept at 37° C. The cells were cultured at 37° C in a CO_2 incubator (air/5% CO_2) for 5 days. The relationship between cell proliferation and the thickness of the coating layer is shown in Table V. On the other hand, the relationship between the thickness of the coating layer and the cell detachment which was observed by a phase contrast microscope when the dishes were transferred from 37° C to room temperature, is also shown in Table V. As shown in Table V, the cell proliferation was excellent independent of the thickness and the cell detachment was improved with the thickness reaching a maximum at a thickness of more than 0.7 um.

For a comparative example, using a non-coated 35 mm plastic dish (Falcon Co.), the human dermal fibroblasts were cultured in the above-mentioned manner. After the cells fully covered the dish, the dish temperature was cooled from 37° C to room temperature, but no detachment of cells was observed. Therefore, the following prior cell detachment process was carried out. The culture medium was discarded from the dish and 2ml of PBS was poured into the dish to wash the surface of the cells for removal of trypsin inhibitor contained in the medium and then the PBS was discarded. Then 2 ml of trypsin/EDTA solution (0.05% trypsin, 0.5 mM EDTA) was added to wash the surface of the cells, the trypsin/EDTA solution was discarded. Again 2 ml of fresh trypsin/EDTA solution was added and the solution except 0.5 ml was discarded. Then the dish was incubated at 37° C for 10 minutes. Using a microscope, complete detachment of cells from the dish was confirmed but the detached cells were isolated from each other and did not make a cell sheet. Also, the prior detachment process using detaching agents consists of a lot of procedures and was significantly complex compared to the current process of merely changing the temperature.

Table V.

Dependency of cell proliferation and cell detachment upon the thickness of coating layer of mixture of PNIPAAm and collagen (1/1)		
Thickness (um)	Cell Proliferation	Cell Detachment
0	◎	×
0.2	◎	○
0.5	◎	○
0.7	◎	◎
0.9	◎	◎
◎ : Excellent ○ : Good × : Poor		

ratio

Example 8

The aqueous solutions of the different mixtures of PNIPAAm and collagen which were used in Example 7 were prepared. The composition ratio of the collagen to PNIPAAm are shown in Table VI. The dishes coated with these aqueous solutions in a thickness of about 0.9 um were prepared by the method similar to Example 7. The human dermal fibroblasts were cultured in a similar manner to Example 7. The relationship of the composition ratio of collagen to PNIPAAm with cell proliferation and cell detachment was measured and is shown in Table VI. The cell proliferation rose with the content of collagen and cell detachment

improved with the content of PNIPAAm. The composition ratio of collagen to PNIPAAm which is suitable to both cell proliferation and detachment, ranges from about 0.1/1.0 to 2.0/1.0.

Table VI

Dependency of cell proliferation and cell detachment upon composition ratio of collagen to PNIPAAm.

Composition Ratio of Collagen to PNIPAAm	Cell Proliferation	Cell Detachment
0.0/1.0	Δ	◎
0.1/1.0	○	◎
0.5/1.0	○	◎
1.0/1.0	◎	◎
2.0/1.0	◎	◎
10.0/1.0	◎	Δ
1.0/0.0	◎	×

◎ : Excellent
○ : Good
Δ : Not Good
× : Poor

Example 9

Using the dish coated with the mixture of collagen and PNIPAAm (1.0/1.0) at a thickness of about 0.9 um in Example 8, the endothelial cells isolated from a calf pulmonary artery (CPAE, American Type Culture Collection) were cultured. The CPAE cells were dispersed in DMEM containing 10% FCS to form a cell density of about 2 x 10^5/ml. Two milliliters of the cell suspension kept at 37° C was poured into the aforesaid coated dish kept at 37° C and the cells were cultured at 37° C in a CO_2 incubator (air/5% CO_2) for 4 days. The cells proliferated and fully covered the dish. When the dish was withdrawn from the 37° C incubator and left at an ambient temperature, it was found by phase contrast microscope examination that the CPAE cells completely detached from the dish surface so as to form a cell sheet.

Example 10

Using the dish coated with the mixture of collagen and PNIPAAm (2.0/1.0) in a thickness of about 0.9 um in Example 8, human epidermal cells (including keratinocytes) were cultured. A thin piece of human skin scraped by a dermatome was treated with trypsin to isolate epidermal cells, which were dispersed in F-12 media supplemented with hydrocortisone and adenine, so as to form a concentration of about 3 x 10^5/ml. Two milliliters of the cell suspension kept at 37° C was poured into the aforesaid coated dish kept at 37° C and the cells were cultured at 37° C in a CO_2 incubator (air/5% CO_2) for 7 days. The medium was replaced every 2 days, using culture medium kept at 37° C. After a 7-day culture, the cells proliferated and fully covered the dish. The outside of the dish was soaked in 10° C water to detach the cells from the bottom of the dish and thus a cell sheet was fabricated. The cell sheet was recovered by merely changing the temperature and the operation was very simple.

Example 11

An aqueous solution (0.5%) of atactic polymethylvinyl ether (Tokyo Kasei K.K.) was prepared. The solution was sterilized by autoclaving (121 °C, 20 minutes) and then cooled to redissolved the polymer. The LCST of the atactic polymethylvinyl ether was about 35 °C in PBS as measured by turbidimetry. This solution was mixed with equal vomume of 0.5% collagen solution used in Example 7 to prepare a solution containing 0.25% polymethylvinyl ether and 0.25% collagen as the final concentration. By a method similar to Example 7, the dish coated with the mixture to a thickness of about 1 um was prepared. Then the human dermal fibroblasts used in Example 3 were dispersed in DMEM to form a cell density of about 2 x 10^5/ml. Two milliliters of the cell suspension kept at 40 °C was poured into the aforesaid dish kept at 40 °C and the cells were cultured at 40 °C in a CO_2 incubator (air/5% CO_2) for 5 days. The cells covered the dish and the dish was withdrawn from the 40 °C incubator and left at an ambient temperature. The cells were spontaneously detached from the dish and the cell sheet was suspended in the medium.

Example 12

An aqueous solution (0.5%) of PNIPAAm which was synthesized in Example 1 was prepared, and sterilized by filtration through a 0.45 micron filter. Then this solution was mixed with an equal volume of 0.05% gelatin aqueous solution (Iwaki Glass K.K.) and 400 ul of the aforesaid mixture solution was poured into a plastic 35 mm cell culture dish (Falcon Co.) and dried in a clean hood at an ambient temperature. The above-mentioned procedure was carried out asceptically. Using the dish coated with the mixture of gelatin and PNIPAAm, the human dermal fibroblasts were cultured by the same method as Example 7. After the cell proliferation, the outside of the dish was cooled to about 10 °C and the cell sheet was able to be detached from the dish and recovered.

Example 13

An aqueous 5% solution of PNIPAAm used in Example 1 was prepared and sterilized by filtration through 0.45 micron filter. Then this solution was mixed with aqueous solutions of fibronectin isolated from calf plasma (Nitta Gelatin K.K.) so as to form mixture solutions with different compositions. The plastic 35 mm dishes (Falcon Co.) were coated with these mixture solutions with different compositions and air-dried at room temperature in a clean hood. These procedures were carried out asceptically. By the same method as Example 8, the human dermal fibroblasts were cultured on the dishes with different compositions and the relationship of the composition of fibronectin and PNIPAAm with cell proliferation and cell detachment was studied (Table VII). As shown in Table VII, cell proliferation improved with the composition ratio of fibronectin to PNIPAAm, but the detachment was poorer than collagen.

Table VII

Dependency of cell proliferation and detachment upon composition ratio of fibronectin to PNIPAAm (thickness: 0.9 um)		
Composition Ratio of Fibrinogen to PNIPAAm	Cell Proliferation	Cell Detachment
0.01/1.0	△	△
0.04/1.0	○	△
0.08/1.0	◎	×
◎ : Excellent ○ : Good △ : Not Good × : Poor		

Example 14

The aqueous solution of PNIPAAm used in Example 13 was mixed with an aqueous solution of adhesive protein isolated from a shellfish, Cell-Tak® (Collaborative Research Inc.) in different compositions. The plastic 35 mm dishes were coated with these mixture solutions to a thickness of about 0.9 µm and by the same method as Example 13 the relationship between the composition ratio of Cell-Tak® to PNIPAAm and cell proliferation and detachment was measured (Table VIII). As shown in Table VIII, the result was almost the same as that of fibronectin although the cell proliferation was slightly inferior to that of fibronectin.

Table VIII

Dependency of cell proliferation and detachment upon composition ratio of Cell-Tak® to PNIPAAm (thickness: 0.9 µm)		
Composition Ratio Cell-Tak® to PNIPAAm	Cell Proliferation	Cell Detachment
0.01/1.0	Δ	Δ
0.04/1.0	Δ	Δ
0.08/1.0	O	×
◎ : Excellent O : Good Δ : Not Good × : Poor		

Example 15

The aqueous solution of PNIPAAm used in Example 13 was mixed with an aqueous solution of one species of lectin, concanavalin A (Hohnen Co.) in different compositions. The plastic 35 mm dishes were coated with these mixture solutions to a thickness of about 0.9 um and by the same method as Example 13, the relationship between the composition ratio of concanavalin A to PNIPAAm and cell proliferation and cell detachment was measured (Table IX). As shown in Table IX, although the cell attachment was observed, no cell proliferation was really recognized.

Table IX

Dependency of cell proliferation and detachment upon composition ratio of concanavalin A to PNIPAAm (thickness: 0.9 um)		
Composition Ratio of Concanavalin A to PNIPAAm	Cell Proliferation	Cell Detachment
0.01/1.0	Δ*	Δ
0.04/1.0	Δ*	Δ
0.08/1.0	Δ*	Δ
0.5 /1.0	○*	Δ

*: Although cell attachment was good, no cell proliferation was really observed.
◎ : Excellent
○ : Good
Δ : Not Good
× : Poor

Example 16

First, using 0.5% aqueous solution of PNIPAAm prepared in Example 1, the plastic 35 mm dish was coated with PNIPAAm to a thickness of about 0.9 um and then 400 ul of aqueous solution of fibronectin (concentration 0.1 mg/ml) kept at 37° C and used in Example 13 was poured into the aforesaid PNIPAAm-coated dish kept at 37° C and the dish was air-dried asceptically in a 37° C incubator. By this method, the dish having a laminated coating composed of a PNIPAAm layer and subsequently of a fibronectin layer whose fibronectin density is about 5 ug/cm^2, was prepared. Using this coated dish, by the same method as Example 7, the cell proliferation and cell detachment profiles were evaluated. As a result, the cell proliferation and simultaneously the cell detachment were excellent.

Example 17

A commercial available polyethylene terephtalate (PET) film (size: 5 cm x 5 cm, thickness: 100 um, Toray Ind. Inc.) was inserted into a chamber of plasma irradiation apparatus with an internal electrode (Samco International K.K.). After the interior of the chamber was evacuated to 0.8 Torr, argon gas was introduced into the chamber at a flow rate of 30ml/min and the film was irradiated with plasma (output = 100 Watts, frequency = 13.56 MHz) for 15 seconds. Immediately thereafter, 5% aqueous solution of NIPAAm monomer was introduced to the chamber so as to soak the film in the solution and the polymerization was carried out at room temperature for 16 hours. In advance, from the monomer solution, contaminated air was completely removed by bubbling with argon. After washing the treated film thoroughly with water, it was dried under vacuum at an ambient temperature.

For controls, without introducing NIPAAm monomer into the chamber, the PET film was treated by plasma irradiation in similar conditions. Also, the PET film merely coated with PNIPAAm was prepared by a solvent casting method using PNIPAAm aqueous solution. The contact angles of the above-mentioned films against water 10° C and 40° C were measured and listed in Table X. As shown in Table X, in untreated film and plasma-treated film, no change in contact angle was observed between at 10° C and at 40° C, but in PNIPAAm coated film and PNIPAAm plasma-grafted film, significant differences in contact angle were found. Both films showed hydrophilicity at 10° C and hydrophobicity at 40° C.

Table X

Contact angles of films		
	Contact Angle (°)	
Film Species	10°C	40°C
Untreated Film	77.0 ± 1.6	77.0 ± 2.3
Plasma-Treated Film	50.0 ± 1.7	50.1 ± 0.5
PNIPAAm Coated Film	43.7 ± 3.4	64.1 ± 2.9
PNIPAAm Plasma-Grafted Film	48.4 ± 4.9	60.7 ± 6.8

Example 18

50 g of NIPAAm, 3.3 g of n-BMA and 1.5 g of N,N'-methylenebisacrylamide were dissolved in 100 ml of water. This solution was suspended in 500 ml of hexane. Using ammonium persulfate 0.13 g and tetramethylethylenediamine 0.13 g as the polymerization initiator, they were polymerized at an ambient temperature for 24 hours in a stream of nitrogen gas with a constant agitation at 300 rpm, to acquire crosslinked microbeads. The microbeads were washed with 15°C cold water, and then dried under vacuum. The average particle size of microbeads in 15°C cold water was 210 microns. When the temperature was raised to 37°C, the beads shrank and became opaque. After washing 1 g of microbeads with sterile water thoroughly, the beads were dispersed in 5 ml of DMEM containing 10% FCS which was warmed to 37°C, and mouse dermal fibroblasts were added to bring the cell concentration to about 1 x 10^7/ml. Then they were transferred into a glass beaker, and incubated at 37°C for 4 hours in a CO_2 gas incubator agitated by a magnetic stirrer. Later, microbeads were removed from the glass beaker, and the surface of the microbeads was washed twice with the PBS which was warmed to 37°C, and then the microbeads were immobilized with 1% glutaraldehyde-PBS which was warmed to 37°C. After washing the immobilized microbeads with distilled water, they were dehydrated with alcohol, dried by the critical point drying technique, and gold palladium was deposited by vapro process. Cell adhesion was observed by scanning electron microscopy. Alternatively, microbeads taken out after 4 hours incubation were allowed to stand for 20 minutes in the 15°C medium, and the medium was discarded. The surface of the microbeads was washed twice with 15°C PBS, and the microbeads were immobilized with the 1% glutaraldehyde-PBS which was cooled to 15°C. The immobilized microbeads were examined by scanning electron microscopy by the same procedure as before, to observe cell adhesion. As a result, it was found that the number of cells on the surface of microbeads immobilized at 15°C was extremely small compared to the number of cells on the surface of microbeads immobilized at 37°C. Almost no cells were found attached to the surface of microbeads immobilized at 15°C. This evidence suggests that cells were detached entirely from the surface of the beads by cooling the microbeads.

Example 19

Using the dish coated with the mixture of collagen and PNIPAAm (collagen/PNIPAAm = 1.0/1.0) at a thickness of about 0.9 um which is prepared in Example 7, the human dermal fibroblasts were cultured in the similar manner to Example 7. After a 5-day culture, the cells fully covered the dish. When the dish was withdrawn from a 37°C incubator and left at an ambient temperature, the cell sheet spontaneously detached from the dish and the suspended cell sheet was washed twice with fresh medium in order to remove dissolved PNIPAAm and collagen from the cell sheet. Thereafter, the cell sheet was transferred to a new non-anchorage hydrophobic 35 mm dish (Falcon Co.) containing 2 ml of fresh culture medium, and was cultured under suspension for 2 days until a spheroidal cell cluster was obtained. The size of the obtained spheroidal cell cluster was about 1 mm and the cell density was in the order of 10^9/ml.

On the other hand, the obtained spheroidal fibroblast cluster was cultured on the non-anchorage dish in a CO_2 incubator (air/5% CO_2) for an extra 20 days and then the cell cluster was transferred to the plastic hydrophilic dish (Falcon Co.) and was cultured in similar conditions to the above-mentioned. After a 2-day culture, the cell cluster reattached to the hydrophilic dish surface and reproliferated after a 10-day culture. This evidence demonstrates that the cell cluster has proliferation activity even after a long preservation.

For controls, using the dish coated only with collagen prepared in Example 8, in a similar manner to the above-mentioned conditions, the human dermal fibroblasts were cultured. In order to detach the cell sheet which fully covered the dish after a 4-day culture, the prior cell detachment procedures were carried out. The old culture medium was discarded from the dish and 2 ml of PBS was poured into the dish to wash the surface of the cells for removal of trypsin inhibitors contained in the medium and then the PBS was discarded. Then 2 ml of trypsin/EDTA solution (0.05% trypsin, 0.53 mM EDTA) was added to wash the surface of the cells, and the trypsin/EDTA solution was discarded. Again 2 ml of fresh trypsin/EDTA solution was added and the solution, except 0.5 ml, was discarded. Then the dish was incubated at 37° C for 10 minutes. Using a microscope, complete detachment of the cells from the dish was confirmed, but the detached cells were isolated from each other and neither made a cell sheet nor a cell cluster. Accordingly, by this cell detachment method, it was impossible to prepare these cell sheets and cell clusters.

Example 20

The fibroblast sheet which was obtained by the detachment procedure in Example 16, was transferred to a non-anchorage hydrophobic dish and cultured in a similar manner to Example 19. After a 2-day culture the cell sheet completely changed to a cluster whose size was about 1 mm. After the cell cluster was preserved on the hydrophobic dish at 37° C in a CO_2 incubator (air/5% CO_2 for 3 months, the cluster was again transferred to a new hydrophilic dish and was cultured. After 2 days, the cluster attached to the dish and started to reproliferate. This evidence shows that the cell cluster continues to live even after 3 months of preservation.

Claims

1. A cell culture substrate comprising a temperature-responsive polymeric compound having a lower LCST than the culture temperature.

2. The cell culture substrate of Claim 1, wherein the said polymeric compound is selected from the group comprising poly-N-substituted acrylamide derivatives, poly-N-substituted methacrylamide derivatives, their copolymers, polyvinylmethyl ether and partially acetylated polyvinylalcohol.

3. The cell culture substrate of Claim 1, further comprising substances which effectively control cell functions.

4. The cell culture substrate of Claim 3 wherein said substance which effectively controls cell function is selected from the group comprising extracellular matrix, gelatin, lectin, anchorage oligopeptide and adhesive protein isolated from shellfish.

5. The cell culture substrate of Claim 4 wherein the said extracellular matrix is selected from the group consisting of collagen, fibronectin, vitronectin, laminin, proteoglycan, glycosaminoglycan and thrombospondin.

6. The cell culture substrate of Claim 1 wherein the said polymeric compound is coated on the surface of the supporting material.

7. The cell culture substrate of Claim 1 wherein the said polymeric compound is graft-copolymerized on the surface of the supporting material.

8. The cell culture substrate of Claim 1 wherein the said polymeric compound has a crosslinked structure.

9. The cell culture substrate of Claim 1 wherein the shape at the cell culture temperature is a film, sheet, particle, fiber, flake, sponge or microbead.

10. The cell culture substrate of Claim 3 wherein said polymeric compound and said substance which effectively controls cell function are coated on the surface of the supporting material as a homogeneous mixture.

11. The cell culture substrate of Claim 3, wherein said polymeric compound and said substance which effectively controls cell function are coated on the surface of the supporting material in a sequential manner.

12. A method for preparation of the cell culture substrate of Claim 3 comprising coating said substance which effectively controls cell function and said temperature-sensitive polymeric compound having lower LCST than the culturing temperature on the surface of the support member.

13. A method for fabricating cell sheets comprising using a cell culture substrate containing at least partially a temperature-responsive polymer having lower LCST than the cell culture temperature, lowering the temperature to lower than LCST after proliferation of cells, and removing the proliferated cells from the supporting material.

14. The method of Claim 13, wherein said temperature-responsive polymer is selected from poly-N-substituted acrylamide derivatives, its copolymers, or partially acetylated polyvinyl alcohols or polymethylvinyl ethers.

15. The method of Claim 13, wherein said cell culture substrate further comprises a substance which effectively controls cell function.

16. Cell sheets prepared by the method of Claim 13.

17. A method for preparation of cell clusters, comprising culturing cells on a substrate containing temperature-sensitive polymeric compound having a lower LCST than at least the cell culturing temperature, and lowering the temperature below the LCST after propagation of cells to release the propagated cells from the substrate.

18. The method of Claim 17, wherein said substrate further comprises a substance which effectively controls cell function.

19. The method of Claim 27, wherein said temperature-sensitive polymeric compound is selected from poly-N-substituted methacrylamide derivatives, their copolymers, polymethylvinyl ethers or partially acetylated polyvinyl alcohols.

20. Cell clusters prepared by the method of Claim 17.

European Patent Office

EUROPEAN SEARCH REPORT

Application Number

EP 90 25 0065

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.5)
Y	PATENT ABSTRACTS OF JAPAN, vol. 8, no. 205 (C-243)[1642], 19th September 1984; & JP-A-59 95 930 (KOGYO GIJUTSUIN (JAPAN)) 02-06-1984 * The entire abstract * ---	1-20	C 12 N 5/00
Y	WO-A-8 808 448 (M. BAY) * The claims; page 2, line 30 - page 3, line 15; page 5, lines 1-17; page 7, lines 30-33; page 9, lines 5-11; page 10, lines 10-29; page 11, lines 3-5; page 12, lines 18-26 * -----	1-20	
			TECHNICAL FIELDS SEARCHED (Int. Cl.5)
			C 12 N

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	26-06-1990	RYCKEBOSCH A.O.A.

CATEGORY OF CITED DOCUMENTS

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